The transcription factor PPARalpha is overexpressed and is associated with a favourable prognosis in IDH-wildtype primary glioblastoma

Running title: **PPARalpha expression in primary glioblastoma**

Haynes HR[1], White P[2], Hares KM[3], Redondo J[3], Kemp KC[3], Singleton WGB[4], Killick-Cole CL[4], Stevens JR[5], Garadi K[6], Guglani S[7], Wilkins A[3], Kurian KM[1]

1. Brain Tumour Research Group, Institute of Clinical Neurosciences, University of Bristol, UK

2. Applied Statistics Group, University of the West of England, Bristol, UK

3. MS and Stem Cell Research Group, Institute of Clinical Neurosciences, University of Bristol, UK

4. Functional Neurosurgery Research Group, Institute of Clinical Neurosciences, University of Bristol, UK

5. Department of Cellular Pathology, North Bristol NHS Trust, Bristol, UK

6. Bristol Haematology and Oncology Centre, University Hospital Bristol Trust, Bristol, UK

7. Gloucestershire Oncology Centre, Gloucestershire Hospitals NHS Foundation Trust, UK

Oct 2016

Word count: 2500 (main body)

Authors report no conflicts of interest

ABSTRACT

Aims: PPARα agonists are in current clinical use as hypolipidaemic agents and show significant antineoplastic effects in human glioblastoma models. To date however, the expression of PPARα in large-scale glioblastoma data sets has not been examined. We aimed to investigate the expression of the transcription factor PPARα in primary glioblastoma, the relationship between PPARα expression and patients' clinicopathological features and other molecular markers associated with gliomagenesis.

Methods and Results: Using protein immuno-blotting techniques and RT-qPCR, PPARα was found to be significantly overexpressed in glioblastoma compared to control brain tissue (p=0.032 and p=0.005). *PPARA* gene expression was found to be enriched in the classical glioblastoma subtype within The Cancer Genome Atlas (TCGA) data set. Although not associated with overall survival when assessed by immunohistochemistry, cross-validation with the TCGA data set and multivariate analyses identified *PPARA* gene expression as an independent prognostic marker for overall survival (p=0.042). Finally, hierarchical clustering revealed novel, significant associations between high *PPARA* expression and a putative set of glioblastoma molecular mediators including *EMX2*, *AQP4* and *NTRK2*.

Conclusions: PPARα protein is overexpressed in primary glioblastoma and high *PPARA* gene expression functions as an independent prognostic marker in the glioblastoma TCGA data set. Further studies are required to explore genetic associations with high *PPARA* expression and to analyse the predictive role of PPARα expression in glioblastoma models in response to PPARα agonists.

INTRODUCTION

Primary glioblastoma has an incidence of 4/100,000 per year and a 3% five year overall survival rate.[1] Glioblastoma is a molecularly heterogeneous cancer [2,3] and it is key that new tools are developed that better delineate its biological variants.[4,5]

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors governing lipid, carbohydrate and amino-acid metabolism.[6] Synthetic PPARα agonists such as fenofibrate and clofibrate are widely used clinically as hypolipidaemic agents. A large study has suggested that the use of fibrates is associated with a reduced probability of death from cancer.[7]

Fenofibrate and clofibrate have been reported to exert anticancer effects in breast cancer models,[8,9] PPARα activation inhibits the growth of non-small cell lung carcinoma cells [10,11] and suppresses tumour growth in ovarian carcinoma models.[12] Conversely, overexpression of PPARα has been reported to predict reduced clinical survival in ovarian carcinoma cohorts .[13]

The role of PPARα in pre-clinical models of high grade glial tumours is not yet fully elucidated. However, fenofibrate exerts tumour suppressive effects via modulation of angiogenesis in a U87 mouse model of high grade glioma (HGG).[14] Fenofibrate decreases the motility of HGG cells *in vitro*,[15] induces G_0/G_1 cell cycle arrest and reduces HGG cell viability,[16] decreases expression of the glioma stem cell marker CD133 [17] and induces BIM-mediated apoptosis.[18] Most recently, fenofibrate has been shown to induce ketogenesis[19] and inhibit glycolysis [20,21] in glioblastoma *in vitro* models.

PPARα is overexpressed in HGG *in vitro* compared to normal human astrocytes [15] and the expression of PPARα correlates with grade of malignancy in glial tumours.[22] An association between hypoxic *in vitro* conditions and increased PPARα expression has been reported.[23] However, to date PPARα expression has not been examined in large-scale glioblastoma cohorts nor its association between key clinicopathological covariates or genetic mediators determined.

In this study we examined the expression of PPARα protein and *PPARA* mRNA in IDH1 (isocitrate dehydrogenase)-wildtype primary human glioblastomas. Wildtype IDH1 status is a key genetic marker in primary glioblastoma.[24,25] We also investigated the relationship between PPARα expression and other clinicopathological factors. Finally we used large scale microarray data to establish previously unreported genetic associations with high *PPARA* expression across the whole transcriptome.

MATERIALS AND METHODS

Study samples

IDH1-wildtype glioblastoma surgical specimens (snap frozen), diagnosed 2010-2013, were obtained from the Brain Tumour Bank Southwest UK. Formalin-fixed paraffin-embedded (FFPE) tissue and clinical data were available for each patient. FFPE tissue from histologically normal anterior temporal lobe resections and frozen post-mortem healthy cortical samples were used as controls. BrainUK ethical approval (14/008, 15/017) and in collaboration with the UK Multiple Sclerosis Tissue Bank.

Protein extraction

Protein was extracted from tissue samples using the Ambion® PARIS™ system (Life Technologies, UK). Protein concentrations were determined using the Qubit® Quant-It protein kit and Qubit® Fluorometer (Thermofisher Scientific, UK).

Western Blotting and immuno dot-blotting

Both western blotting and immune dot blotting were carried out as previously described.[26] Primary antibodies were PPARα (1:2000 Abcam-ab8934) and GAPDH (1:10,000 Abcam-ab9484). Secondary anti-rabbit/anti-mouse horseradish peroxidise-conjugated antibodies (Abcam) were used to detect immunoreactivity. Protein expression was visualised using chemiluminescence (Amersham ECL™) with the Biorad Universal III Bioplex Imager (Biorad, UK). Densitometric analysis of protein dots was performed using ImageLab software (Biorad).

FFPE RNA extraction and RT-qPCR

Total RNA was extracted from either whole control tissue (n=17; x10 5μm FFPE unstained sections) or macro-dissected regions of glioblastoma (n=48; x10 5μm FFPE unstained sections) using the Omega EZNA.FFPE RNA spin column kit (OmegaBio-Tek, USA) including a gDNA elimination step. RNA purity and quantification was determined using a Nanodrop1000. RNA was reverse transcribed to cDNA using the Clontech TaKaRa PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, USA). Real time

quantitative PCR was performed using a Step One Plus instrument with Taqman® Fast Gene Expression Mastermix (both Applied Biosytems) and assay on demand (AoD) gene expression products for *PPARA* (Hs00947536_m1) (amplicon length 62bp) and *GAPDH* [27] (Hs02758991_g1) (amplicon length 93bp) (Taqman® MGB probes, FAM dye-labelled, Applied Biosytems). The efficiency and linear range for both AoD were determined using a dilution series standard curve prior to expression analysis of the cohort.[28] Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.[29]

MGMT promoter methylation status and IDH1 R132H mutation analysis

As part of the standard diagnostic assessment, *MGMT* promoter methylation was determined by methylation sensitive PCR [30] and IDH1R132H immunohistochemistry was performed.[31]

Automated Immunohistochemistry (IHC)

All stages of immunohistochemistry were performed using the Leica Bond autostaining platform and a Bond Polymer Refine Detection kit (Leica, IL, USA) as previously described [32] 2μm sections were incubated with Bond epitope retrieval solution 1 (pH 6.0, 30 minutes) and PPARα antibody (1:55 Abcam-ab8934) followed by the standard autostainer procedures. Sections from PPARα high/low expressing tumours established using immunoblotting were used as positive/negative controls for IHC antibody work up. Primary antibody omission controls were included in each IHC run. 17 control and 100 glioblastoma samples were stained for PPARα.

The immunohistochemical expression of PPARα was quantified using a double-blind system (Table 1). Addition of the intensity and extensivity scores gave a composite score. A score of ≥5 was considered high PPARα expression. Assessments were conducted independently by researchers blinded to the clinical data (H.R.H and K.M.K). Discrepant evaluations between researchers (difference in composite score ≥3 or difference in high vs low outcome score) triggered a consensus meeting and final expression score agreement.

TCGA data set gene expression analysis

The data was pre-processed using lowness normalisation, log-transformed and the mean used to calculate gene level summaries.[33] The data was accessed and processed via the GlioVis online platform (http://gliovis.bioinfo.cnio.es). Differential expression analysis was performed using eBayes and lmFit functions of the 'limma' package with a log2 fold change of 1 and p-value of 0.05.[34] Pearson correlation analysis was applied to significantly differentially expressed genes. Cross-validation with the Rembrandt glioblastoma data set [35] was carried out. Gene nomenclature was cross-referenced against the NCBI database (https://www.ncbi.nlm.nih.gov/gene/) and all gene aliases were used in subsequent literature review.

Statistical Analysis

Mann-Whitney test was used to compare control vs disease protein/mRNA expression. Fisher's exact test was used to test possible associations between PPARα expression and clinicopathological covariates. Overall survival (OS) was defined as the interval between the date of diagnosis and the date of death. Kaplan-Meier survival curves were compared

7

with the log-rank test. Hazard ratios and 95% confidence intervals were calculated by univariate and multivariate Cox proportional hazards regression. All statistical tests were two-tailed. Differences at $p<0.05$ were considered significant. Statistical tests carried out using GraphPad v5 (GraphPad, USA), IBM SPSS Statistics22 software (IBM, USA) and the GlioVis tool (http://gliovis.bioinfo.cnio.es).

RESULTS

PPARα protein and *PPARA* gene expression is increased in IDH1-wildtype glioblastoma

The primary antibodies used throughout this study were validated for antibody specificity using western blotting (Figure 1A). There was a significant increase in PPARα protein expression in the glioblastoma samples (p=0.032) (Figure 1B). RT-qPCR showed a 2.03 fold increase in *PPARA* mRNA expression in the glioblastoma samples compared to controls (p=0.005) (Figure 2).

PPARA gene expression is differentially enriched in transcriptomic glioblastoma subtypes

PPARA transcript expression is significantly increased in the classical glioblastoma subtype in both the TCGA (p=0.001) (Figure 3A) and Rembrandt (p=0.05) (Figure 3B) data sets when compared to proneural subtype. Significantly decreased expression is noted in the mesenchymal subtype within the Rembrandt data set (p=0.002) (Figure 3B), an effect not found in the TCGA data.

Characterisation of PPARα IHC expression

The control tissue showed neuronal PPARα cytoplasmic and variable nuclear positivity in cortical grey matter (Figure 4 A&B). A negative white matter immunophenotype was seen. Where the scoring criteria established a high expression, a nuclear or mixed cytoplasmic and nuclear immunohistochemical expression pattern was seen (Figure 4 E&F). In high expressing tumours, PPARα intratumoral heterogeneity was observed as admixed negative cells (Figure 4E).

Associations between PPARα IHC score and clinicopathological variables

The clinicopathological features of the glioblastoma cohort (n=100) are summarized in Table 2. The mean age at diagnosis was 61years. PPARα IHC scores were high in 60 and low in 40 glioblastoma samples. There was no significant association between PPARα IHC score and patient sex, patient age, Karnofsky Performance Score (KPS), tumour location and *MGMT* promoter methylation status (p>0.05 for all covariates) (Table 2).

Association between PPARα IHC score and OS

Univariate Cox's proportional hazards analysis revealed no prognostic role for patient sex, age, KPS or *MGMT* promoter methylation status (Table 3). A significantly reduced OS was seen for those with diffuse/multifocal/thalamic glioblastoma (median OS: 4 months). OS

was significantly associated with surgical resection and adjuvant treatment. In this cohort, there was no prognostic role for PPARα expression by IHC.

Associations between TCGA *PPARA* gene expression and clinicopathological variables

The clinicopathological features of the TCGA glioblastoma cohort (n=473) are summarized in Table 4. The mean age at diagnosis was 59.7years. There was no significant association between *PPARA* gene expression and patient age or *MGMT* promoter methylation status (p>0.05) (Table 4).

***PPARA* gene expression is associated with OS in the TCGA dataset**

Univariate Cox's proportional hazards analysis for OS revealed a significant prognostic role for age, *MGMT* methylation and adjuvant treatment (Table 5). In this cohort, patients with high *PPARA* gene expression had a statistically significant increase in OS (upper quartile vs other 3 quartiles, median OS: 15.1 vs 13.6 months; log-rank p value=0.016) (Figure 5A). Analysis of classical glioblastoma only (as a model of almost exclusively IDH1-wildtype glioblastoma) additionally showed a significant increase in OS (upper quartile vs other 3 quartiles, median OS: 16.6 vs 14.0 months; log-rank p value=0.006) (Figure 5B).

***PPARA* gene expression is an independent prognostic biomarker**

168 missing values for *MGMT* status were observed in the TCGA data (Tables 4 & 5). We found that missing *MGMT* values were significantly associated with OS and *PPARA*

expression (p<0.001; both). In order to accommodate these missing data, multiple imputation was performed for missing *MGMT* values before multivariate analysis. When a single extreme outlier in the TCGA data was excluded (OS = 127.6 months), this multivariate model indicated that the prognostic value of *PPARA* expression was independent of age, *MGMT* methylation status and adjuvant treatment (p=0.042) (Table 6).

PPARA gene expression has significant genetic correlations in the TCGA dataset

The TCGA dataset was interrogated to determine genetic associations with the prognostically significant high *PPARA* expression. Differential gene expression analysis revealed gene subsets clustering with high (n=39) and low (n=31) *PPARA* expression (Figure 6). Of the gene expression values clustered with high *PPARA* expression, 10 have previously been associated with primary glioblastoma in published studies. Each of these 10 genes was significantly positively correlated with *PPARA* when analysed across all samples in the TCGA data set (n=489). Of this group, 5 transcripts remained correlated when cross-referenced against the Rembrandt data set (n=217) [35] (Table 7, Figure 7).

DISCUSSION

In this study we examined the expression of PPARα in IDH-wildtype glioblastoma,[36] defined clinically as primary glioblastoma.[37] PPARα agonists may have considerable advantages as repurposed antineoplastic agents for glioblastoma including good tolerance in chronic administration and a low side effect profile. Indeed, fenofibrate intracranial drug delivery methods are currently in development.[38]

Our results indicate that the expression of PPARα protein and *PPARA* mRNA is significantly increased in glioblastoma. Whether this overexpression is an early or initiating event in the malignant transformation of glioma stem cells,[39,40] is essential for on-going tumour progression or contributes to adjuvant therapy response,[41] remains to be elucidated.

We also report a pattern of mixed cytoplasmic and nuclear PPARα expression by IHC in the glioblastoma tissue. Such a mixed pattern of protein localisation is consistent with the ligand activated transcription factor function of PPARα and has previously been reported *in vitro.*[15]

In the present study, significant associations between OS and patient age, tumour location, *MGMT* methylation, extent of surgical resection and adjuvant treatment were seen. This is consistent with published data.[42,43] We also reported that PPARα expression by IHC showed no prognostic significance, although the sample size was limited. However, post hoc interrogation of the TCGA data set showed high expression of *PPARA* was independently prognostically significant. This model utilised a multiple imputation approach for missing *MGMT* values to avoid statistical bias which may be caused by excluding missing data.[44] This model also excluded a single outlier. No root cause could be found for this outlier as an inconsistent observation and we provided multivariate models with and without its inclusion.[45] It is of note that this outlying OS value may reduce the power of future TCGA analyses. Further work is needed examining the OS advantage of PPARα by IHC in a larger (prospectively collated) clinical cohort and whether PPARα may have translational relevance as a prognostic marker in diagnostic practice.

We have demonstrated enrichment for *PPARA* expression in classical glioblastoma, compared to proneural subtypes in both the TCGA and Rembrandt data sets. Secondary *IDH*-mutant glioblastoma cluster in the proneural subtype and show a unique epigenetic phenotype of global DNA hypermethylation.[46,47] Primary glioblastoma, lacking *TP53* and *IDH1/2* mutations are defined as having a classical gene signature [33,48] and are of interest in this study. The increased expression of *PPARA* in classical vs mesenchymal glioblastoma, seen in the Rembrandt data set alone, suggests that recurrent tumours, with a mesenchymal type gene signature,[49,50] have decreased levels of this transcript. Studies examining the genetic mechanisms mediating increased expression, demonstrated herein, as well as post treatment (recurrent tumour) expression are warranted.

Although PPARα signalling has been associated with a variety of malignancies, the precise role of neoplastic PPARα expression remains to be elucidated. In this study we used differential gene expression analysis to determine that 5 genes previously associated with gliomagenesis are correlated with high *PPARA*. Of particular interest is the correlation between high *PPARA* and *EGFR*. Glioblastomas with EGFR amplification or overexpression cluster in the classical expression subtype.[33,51] It has recently been shown that PPARα enhances the transcription of *EGFR*.[52] However, the prognostic significance of EGFR overexpression in glioblastoma is uncertain.[53–55] Pre-clinical investigation of the antineoplastic effects of combined PPARα agonism and EGFR kinase inhibitors would be a logical extension to this study.

EMX2, a transcription factor with key neurodevelopmental roles,[56] reported here as correlating with high *PPARA* expression, may function as a tumour suppressor in glioblastoma models.[57] The transcription factor *NPAS3* has similarly been implicated in

neurodevelopment [58,59] and its knockdown induces aggressive anaplastic astrocytomas in xenograft models.[60] Demonstrated herein, *NPAS3* expression additionally correlates with *PPARA*, although its function in primary glioblastoma has not, to date, been reported. Also significantly correlated by expression was the kinase *NTRK2* which has been shown to correlate with improved glioma survival.[61] Conversely, *AQP4* has been associated with anti-apoptotic [62] and pro-invasive effects.[63] Further work is required to determine the role of each of these molecular markers in the high *PPARA* expressing subgroup with improved OS that we have described.

In summary, our study showed that PPARα is significantly overexpressed in primary glioblastoma. Interrogation of the TCGA data set has revealed an independent prognostic role for *PPARA* expression and significant correlation with a set of glioblastoma-associated regulators. Additional studies are required to determine whether a PPARα protein or gene expression signature has predictive value for PPARα agonists used as a novel therapy for patients with glioblastoma.

Figure 1: PPARα protein expression in control and glioblastoma tissues. **(a)** Western blot validation of antibodies used in immunoblotting experiments and immunohistochemistry. **(b)** PPARα protein expression was examined in post-mortem GM and WM control tissue samples (n=4 GM; n=4 WM) and glioblastoma (IDH1-wildtype) patient samples (n=28). The test statistic is Mann Whitney test; 2 tailed p value. Error bars show standard error of the mean. *p<0.05. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GM, grey matter; WM, white matter.

Figure 2: *PPARA* expression by RT-qPCR. *PPARA* mRNA expression was examined in FFPE samples of control (histologically normal) cortex (n=17) and glioblastoma (IDH1-wildtype) (n=48) by RT-qPCR. The geometric mean and 95% confidence interval are shown on a logarithmic scale (to base2). The test statistic is Mann Whitney test; 2 tailed p value. **p<0.01; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 3: *PPARA* expression in transcriptome data sets. (a) TCGA data set analysis (classical n=182 [37.2%], mesenchymal n=156 [31.9%], proneural n=151 [30.9%]). **(b)** Rembrandt data set analysis (classical n=79 [36.1%], mesenchymal n=70 [31.9%], proneural n=70 [31.9%]). In the box plots the upper and lower "hinges" correspond to the 25th and 75th percentiles. The upper/lower whisker extends to the highest/lowest value that is within 1.5 * IQR (inter-quartile range). Data beyond the end of the whiskers are outliers. The test statistic is Tukey's honest significant differences. Normalised and log transformed mRNA gene level summaries shown. **p<0.01; *p<0.05; ns, not significant. IDH1 mutation status not available for the Rembrandt data set. Data analysis carried out using GlioVis online tool.

Figure 4: Representative PPARα expression by IHC. (a) and **(b)** control tissue showed cytoplasmic and some nuclear positivity in pyramidal neurones within the grey matter. **(c)** and **(d)** low expression of PPARα in glioblastomas samples ranged from no expression (c) to some weak, predominately cytoplasmic expression (d). **(e)** and **(f)** high expression of PPARα. Negative staining regions in (f) represent microvascular proliferations. GM, grey matter. Scale bar = 100µm.

Figure 5: **Survival analysis for glioblastoma patients.** Total TCGA data set analysed. **(a)** Kaplan-Meier plot of overall survival from TCGA data set - *PPARA*

expression vs survival: across all glioblastoma subtypes. **(b)** Kaplan-Meier plot of overall survival from TCGA data set - *PPARA* expression vs survival: restricted to classical glioblastoma subtypes. Normalised and log transformed mRNA gene level summaries shown. **p<0.01; *p<0.05. HR; hazard ratio (95% CI). Data analysis carried out using GlioVis online tool.

Figure 6: Hierarchical clustering analysis. A heatmap displaying 70 differentially expressed genes. Up-regulated genes (at least a 2-fold increase in gene expression) have positive values and are displayed red. The lower yellow bar represents the low *PPARA* expressing samples, the blue bar the high *PPARA* expressing samples. Columns represent the patient samples. Rows represent individual differentially expressed genes. The spread of IDH1 mutations and *MGMT* promoter methylation status can be seen in the upper coloured bars.

Figure 7: Analysis of *PPARA*-correlated genes. Genes previously associated with gliomagenesis and revealed by hierarchical clustering to be differentially expressed with high *PPARA* were examined by Pearson correlation analysis in paired samples in the TCGA microarray data set (n=489) and cross-validated with the Rembrandt microarray data set (n=217). Results from correlations within the TCGA data set are shown. All correlations are p<0.001 and Pearson r values are expressed for each correlation. The 95% confidence interval is represented by the grey shaded area in each plot. The test statistic is Pearson's product moment correlation.

Intensity	Score	Percentage Section	Score
NO STAIN	0	NO STAIN	0
FAINT	1	1-25%	1
MODERATE	2	26-50%	2
STONG	3	51-75%	3
		76-100%	4

Table 1: IHC scoring system for PPARα expression.

For both scores, any surrounding histologically normal cortex or necrotic regions were excluded from analysis.

Clinicopathological variable	Patients (n=100)	PPARα high	PPARα low	p-value
Sex				0.828
Male	68	40	28	
Female	32	20	12	
Age (years)				0.224
<61	52	28	24	
≥61	48	32	16	
KPS *				0.061
≥80	65	42	23	
<80	18	7	11	
Tumour Location **				0.474
Frontal	25	13	12	
Non-frontal/diffuse/multifocal	67	42	25	
***MGMT* promoter methylation ***				0.543
Methylated	49	26	23	
Unmethylated	49	32	17	

Table 2: Association between PPARα expression by IHC and clinicopathological features of IDH1-wildtype glioblastoma.

The test statistic is Fisher's exact test; 2 tailed p value. No significant associations as reported. KPS, Karnofsky Performance Score

* 17 missing data points
** 8 missing data points
*** 2 missing data points

Factor	n (events)	Median OS (months) [95% CI]	p-value (log rank)	Hazard Ratio [95% CI]
Sex			0.149	
Male	68 (53)	12.0 [10.64, 13.36]		1
Female	32 (34)	10.0 [4.04, 15.96]		1.36 [0.88, 2.09]
Age			0.233	
28 – 55	32 (29)	13.0 [10.74, 15.27]		1
56 – 65	32 (27)	11.0 [7.67, 14.33]		1.43 [0.85, 2.42]
66 – 83	36 (31)	11.0 [7.08, 14.92]		1.48 [0.89, 2.46]
KPS *			0.429	
≤80	33 (29)	12.0 [5.25, 18.75]		1
81 – 90	33 (25)	12.0 [10.75, 13.25]		0.82 [0.48, 1.39]
91 - 100	23 (18)	15.0 [13.45, 16.55]		0.69 [0.38, 1.25]
Localisation **			**0.003**	
Frontal	25 (19)	13.0 [1.58, 24.42]		1
Non-frontal	61 (49)	13.0 [11.09, 14.91]		1.03 [0.60, 1.75]
Diffuse[+]	6 (6)	4.0 [0.00, 8.80]		4.07 [1.57, 10.59]
Resection ***			**0.041**	
Partial	53 (44)	11.0 [6.54, 15.46]		1
Complete	39 (29)	14.0 [11.38, 16.62]		0.62 [0.39, 0.99]
MGMT			0.171	
Methylated	49 (37)	13.0 [9.08, 16.92]		1
Unmethylated	49 (43)	12.0 [10.05, 13.95]		1.35 [0.87, 2.11]
Treatment ****			**<0.001**	
Ɨ RT + TMZ full	39 (24)	24.0 [4.64, 43.36]		1
¥ RT + TMZ partial	24 (24)	9.0 [7.21, 10.79]		3.89 [2.12, 7.13]
RT alone	4 (4)	1.0 [----]		24.43 [7.24, 82.40]
Palliative	11 (10)	2.0 [----]		7.13 [3.31, 15.38]
PPARα IHC			0.596	
High	60 (46)	12.0 [9.13, 14,87]		1
Low	40 (35)	12.0 [9.05, 14.95]		0.89 [0.57, 1.39]

Table 3: Overall survival vs. clinical covariates and PPARα IHC expression (n=100)

In this cohort (n=100), the median overall survival (OS) for all patients was 10 months (range: 1 – 58 months). The OS for all patients was 53% at 1 year, 26% at 2 years and 21% at 3 years. Patients who had undergone a partial resection or biopsy only were more likely to receive no adjuvant therapy (Pearson Chi-squared test: p=0.022). Under a Holm-Bonferroni correction for multiplicity of tests the above significant effects remain statistically significant except for 'Resection'. KPS, Karnofsky Performance Score; RT, radiotherapy; TMZ, temozolomide. The bold denotes statistical significance.

[+] Includes multifocal and thalamic tumours
Ɨ Includes full 60Gy (30) plus concurrent TMZ with full 6 cycles adjuvant TMZ
¥ Includes full 60Gy (30) plus concurrent TMZ without full 6 cycles adjuvant TMZ **OR**
Includes full 60Gy (30) without concurrent TMZ but with full 6 cycles adjuvant TMZ

* 11 missing data points
** 8 missing data points
*** 8 missing data points

**** 22 missing data points

Clinicopathological variable	Patients (n=473)	*PPARA* high	*PPARA* low	p-value
Age (years)				0.626**
≤55	161	85	76	
56-64	140	72	68	
≥65	172	82	90	
MGMT **promoter methylation ***				0.063
Methylated	141	69	72	
Unmethylated	164	62	102	

Table 4: Association between *PPARA mRNA expression* and clinicopathological features of IDH1-wildtype glioblastoma.

Recurrent tumours and IDH-mutant tumours were excluded from this TCGA data set analysis. The test statistic is Fisher's exact test; 2 tailed p value. No significant associations as reported.

* 168 missing data points
** The test statistic is Freeman-Halton extension of the Fisher exact probability test

Factor	n	Median OS (months) [95% CI]	p-value (log-rank)	Hazard Ratio [95% CI]
Age			**<0.001**	
11 – 55	161	14.7 [12.96, 16.49]		1
56 – 65	140	12.9 [10.91, 14.98]		1.36 [1.08, 1.72]
66 – 83	172	7.5 [5.50, 9.50]		2.27 [1.82, 2.85]
MGMT *			**0.003**	
Methylated	141	12.2 [9.72, 14.68]		1
Unmethylated	164	10.2 [8.75, 11.65]		1.42 [1.13, 1.79]
Treatment **			**<0.001**	
ǂ RT + TMZ full	193	13.8 [12.64, 14.96]		1
¥ RT + TMZ partial	118	14.9 [13.18, 16.62]		0.74 [0.59, 0.94]
RT alone	135	5.4 [3.96, 6.84]		2.16 [1.73, 2.70]
Palliative	10	3.6 [1.43, 5.77]		3.70 [1.95, 7.03]

Table 5: Overall survival vs. clinical covariates in the TCGA data set (n=473)

Recurrent tumours and IDH-mutant tumours were excluded from this TCGA data set analysis. In this data set, the median overall survival (OS) for all patients was 14.9 months from the date of diagnosis (range: 0.1 – 127.6 months). The OS for all patients was 48.4% at 1 year, 15.6% at 2 years and 7.0% at 3 years. Patient age, *MGMT* methylation status and adjuvant treatment modality were available covariates in the TCGA data. Death occurred in all patients in this data set. Under a Holm-Bonferroni correction for multiplicity of tests the above significant effects remain statistically significant. RT, radiotherapy; TMZ, temozolomide. The bold denotes statistical significance.

ǂ Includes full 60Gy (30) plus concurrent TMZ with full 6 cycles adjuvant TMZ
¥ Includes full 60Gy (30) plus concurrent TMZ without full 6 cycles adjuvant TMZ **OR**
Includes full 60Gy (30) without concurrent TMZ but with full 6 cycles adjuvant TMZ

* 168 missing data points
** 17 missing data points

Factor [a]	p-value	Overall survival Hazard Ratio [95% CI]	Factor [b]	p-value	Overall survival Hazard Ratio [95% CI]
PPARA	0.067	0.58 [0.32, 1.04]	*PPARA*	**0.042**	0.55 [0.31, 0.98]
Age	**<0.001**	1.02 [1.01, 1.03]	**Age**	**<0.001**	1.02 [1.01 - 1.03]
MGMT Methylated Unmethylated	**0.041**	1 1.32 [1.01, 1.73]	**MGMT** Methylated Unmethylated	**0.002**	1 1.05 [1.15 – 1.88]
Treatment * ɫ RT + TMZ full ¥ RT + TMZ partial RT alone Palliative	**<0.001**	1 0.77 [0.61, 0.97] 2.02 [1.61, 2.55] 3.01 [1.57, 5.79]	**Treatment *** ɫ RT + TMZ full ¥ RT + TMZ partial RT alone Palliative	**<0.001**	1 0.81 [0.64 – 1.02] 2.04 [1.63 – 2.58] 2.99 [1.54 – 5.78]

Table 6: Multivariate analysis of factors associated with overall survival in the TCGA data set (n=473[a]; n=472[b])

Recurrent tumours and IDH-mutant tumours were excluded from this TCGA data set analysis. Multiple imputation was performed 1000 times for 168 missing MGMT values before multivariate analysis. (a) model with single OS outlier included. (b) model with single OS outlier excluded. *PPARA* mRNA values and age are expressed as continuous variables. RT, radiotherapy; TMZ, temozolomide.   The bold denotes statistical significance.

ɫ Includes full 60Gy (30) plus concurrent TMZ with full 6 cycles adjuvant TMZ
¥ Includes full 60Gy (30) plus concurrent TMZ without full 6 cycles adjuvant TMZ   **OR**
Includes full 60Gy (30) without concurrent TMZ but with full 6 cycles adjuvant TMZ

 * 17 missing data points

22

PPARA vs gene	TCGA dataset, Pearson's r (95% CI)	Rembrant dataset, Pearson's r (95% CI)
EGFR	0.24 (0.15-0.32)	0.37 (0.25-0.48)
EMX2	0.28 (0.20-0.36)	0.28 (0.15-0.40)
AQP4	0.27 (0.19-0.35)	0.34 (0.21-0.45)
NPAS3	0.35 (0.27-0.42)	0.21 (0.08-0.33)
NTRK2	0.30 (0.21-0.38)	0.30 (0.21-0.38)

Table 7: Correlation between *PPARA* and selected differentially expressed gene mRNA values in paired samples in the TCGA (n=487) and Rembrandt (n=217) data sets.

The test statistic is Pearson's product moment correlation. All correlations have a p-value <0.001.

Acknowledgements:

Author contributions:

Study conception and design: HRH, KMH, KCK, JRS, AW, KMK
Data collection, analysis and interpretation: HRH, PW, KMH, KCK, WGBS, CKC, KG, SG, AW
Manuscript production: HRH, PW, KMH, JR, KCK, CKC, AW, KMK
Final approval of manuscript: HRH, PW, AW, KMK

REFERENCES

1   Ohgaki H, Kleihues P. Epidemiology and etiology of gliomas. Acta Neuropathol 2005;

109: 93–108.

2   Patel AP, Tirosh I, Trombetta JJ, Shalek AK, Gillespie SM, Wakimoto H et al. Single-

cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. Science

2014; 344: 1396–401.

3   Sottoriva A, Spiteri I, Piccirillo SGM, Touloumis A, Collins VP, Marioni JC et al.

Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary

dynamics. Proc Natl Acad Sci U S A 2013; 110: 4009–14.

4   Haynes HR, Camelo-Piragua S, Kurian KM. Prognostic and predictive biomarkers in

adult and pediatric gliomas: toward personalized treatment. Front Oncol 2014; 4: 47.

5   Johnson DR, Galanis E. Incorporation of prognostic and predictive factors into glioma

clinical trials. Curr Oncol Rep 2013; 15: 56–63.

6   Ellis HP, Kurian KM. Biological Rationale for the Use of PPARγ Agonists in

Glioblastoma. Front Oncol 2014; 4: 52.

7   Gardette V, Bongard V, Dallongeville J, Arveiler D, Bingham A, Ruidavets J-B et al.

Ten-year all-cause mortality in presumably healthy subjects on lipid-lowering drugs

(from the Prospective Epidemiological Study of Myocardial Infarction [PRIME]

prospective cohort). Am J Cardiol 2009; 103: 381–6.

8   Chandran K, Goswami S, Sharma-Walia N. Implications of a peroxisome proliferator-

activated receptor alpha (PPARα) ligand clofibrate in breast cancer. Oncotarget 2016;

7: 15577–99.

9   Li T, Zhang Q, Zhang J, Yang G, Shao Z, Luo J et al. Fenofibrate induces apoptosis of triple-negative breast cancer cells via activation of NF-κB pathway. BMC Cancer 2014; 14: 96.

10   Hann SS, Zheng F, Zhao S. Targeting 3-phosphoinositide-dependent protein kinase 1 by N-acetyl-cysteine through activation of peroxisome proliferators activated receptor alpha in human lung cancer cells, the role of p53 and p65. J Exp Clin Cancer Res 2013; 32: 43.

11   Skrypnyk N, Chen X, Hu W, Su Y, Mont S, Yang S et al. PPARα activation can help prevent and treat non-small cell lung cancer. Cancer Res 2014; 74: 621–31.

12   Shigeto T, Yokoyama Y, Xin B, Mizunuma H. Peroxisome proliferator-activated receptor alpha and gamma ligands inhibit the growth of human ovarian cancer. Oncol Rep 2007; 18: 833–40.

13   Davidson B, Hadar R, Stavnes HT, Trope' CG, Reich R. Expression of the peroxisome proliferator-activated receptors-alpha, -beta, and -gamma in ovarian carcinoma effusions is associated with poor chemoresponse and shorter survival. Hum Pathol 2009; 40: 705–13.

14   Panigrahy D, Kaipainen A, Huang S, Butterfield CE, Barnés CM, Fannon M et al. PPARalpha agonist fenofibrate suppresses tumor growth through direct and indirect angiogenesis inhibition. Proc Natl Acad Sci U S A 2008; 105: 985–90.

15   Drukala J, Urbanska K, Wilk A, Grabacka M, Wybieralska E, Del Valle L et al. ROS accumulation and IGF-IR inhibition contribute to fenofibrate/PPARalpha -mediated inhibition of glioma cell motility in vitro. Mol Cancer 2010; 9: 159.

16   Han D-F, Zhang J-X, Wei W-J, Tao T, Hu Q, Wang Y-Y et al. Fenofibrate induces G0/G 1 phase arrest by modulating the PPARα/FoxO1/p27(kip) pathway in human

glioblastoma cells. Tumour Biol 2015; 36: 3823-9.

17   Binello E, Mormone E, Emdad L, Kothari H, Germano IM. Characterization of fenofibrate-mediated anti-proliferative pro-apoptotic effects on high-grade gliomas and anti-invasive effects on glioma stem cells. J Neurooncol 2014; 117: 225–34.

18   Wilk A, Urbanska K, Grabacka M, Mullinax J, Marcinkiewicz C, Impastato D et al. Fenofibrate-induced nuclear translocation of FoxO3A triggers Bim-mediated apoptosis in glioblastoma cells in vitro. Cell Cycle 2012; 11: 2660–71.

19   Grabacka MM, Wilk A, Antonczyk A, Banks P, Walczyk-Tytko E, Dean M et al. Fenofibrate Induces Ketone Body Production in Melanoma and Glioblastoma Cells. Front Endocrinol (Lausanne) 2016; 7: 5.

20   Wilk A, Wyczechowska D, Zapata A, Dean M, Mullinax J, Marrero L et al. Molecular mechanisms of fenofibrate-induced metabolic catastrophe and glioblastoma cell death. Mol Cell Biol 2015; 35: 182–98.

21   Han D, Wei W, Chen X, Zhang Y, Wang Y, Zhang J et al. NF-κB/RelA-PKM2 mediates inhibition of glycolysis by fenofibrate in glioblastoma cells. Oncotarget 2015; 6: 26119–28.

22   Benedetti E, Galzio R, Laurenti G, D'Angelo B, Melchiorre E, Cifone MG et al. Lipid metabolism impairment in human gliomas: expression of peroxisomal proteins in human gliomas at different grades of malignancy. Int J Immunopathol Pharmacol; 23: 235–46.

23   Laurenti G, Benedetti E, D'Angelo B, Cristiano L, Cinque B, Raysi S et al. Hypoxia induces peroxisome proliferator-activated receptor α (PPARα) and lipid metabolism peroxisomal enzymes in human glioblastoma cells. J Cell Biochem 2011; 112: 3891–901.

24   Balss J, Meyer J, Mueller W, Korshunov A, Hartmann C, von Deimling A. Analysis of the IDH1 codon 132 mutation in brain tumors. Acta Neuropathol 2008; 116: 597–602.

25   Nobusawa S, Watanabe T, Kleihues P, Ohgaki H. IDH1 mutations as molecular signature and predictive factor of secondary glioblastomas. Clin Cancer Res 2009; 15: 6002–7.

26   Redondo J, Hares K, Wilkins A, Scolding N, Kemp K. Reductions in kinesin expression are associated with nitric oxide-induced axonal damage. J Neurosci Res 2015; 93: 882–92.

27   Grube S, Göttig T, Freitag D, Ewald C, Kalff R, Walter J. Selection of suitable reference genes for expression analysis in human glioma using RT-qPCR. J Neurooncol 2015; 123: 35–42.

28   Walter RFH, Mairinger FD, Wohlschlaeger J, Worm K, Ting S, Vollbrecht C et al. FFPE tissue as a feasible source for gene expression analysis--a comparison of three reference genes and one tumor marker. Pathol Res Pract 2013; 209: 784–9.

29   Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402–8.

30   Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 2000; 343: 1350–4.

31   Kurian KM, Haynes HR, Crosby C, Hopkins K, Williams M. IDH mutation analysis in gliomas as a diagnostic and prognostic biomarker. Br J Neurosurg 2013; 27: 442–5.

32   Zhou S, Venkatramani R, Gomulia E, Shillingford N, Wang L. The diagnostic and prognostic value of SALL4 in hepatoblastoma. Histopathology 2016; 69:822-830

33   Verhaak RGW, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD et al. Integrated

genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 2010; 17: 98–110.

34   Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 2015; 43: e47.

35   Madhavan S, Zenklusen J-C, Kotliarov Y, Sahni H, Fine HA, Buetow K. Rembrandt: helping personalized medicine become a reality through integrative translational research. Mol Cancer Res 2009; 7: 157–67.

36   Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol 2016; 131: 803–20.

37   Ohgaki H, Kleihues P. The definition of primary and secondary glioblastoma. Clin Cancer Res 2013; 19: 764–72.

38   Grabacka M, Waligorski P, Zapata A, Blake DA, Wyczechowska D, Wilk A et al. Fenofibrate subcellular distribution as a rationale for the intracranial delivery through biodegradable carrier. J Physiol Pharmacol 2015; 66: 233–47.

39   Pollard SM, Yoshikawa K, Clarke ID, Danovi D, Stricker S, Russell R, et al. Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. Cell Stem Cell. 2009; 4: 568–580.

40   Lee J, Kotliarova S, Kotliarov Y, Li A, Su Q, Donin NM et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 2006; 9: 391–403.

41   Chen J, Li Y, Yu T-S, McKay RM, Burns DK, Kernie SG et al. A restricted cell

population propagates glioblastoma growth after chemotherapy. Nature 2012; 488: 522–6.

42   Ostrom QT, Bauchet L, Davis FG, Deltour I, Fisher JL, Langer CE et al. The epidemiology of glioma in adults: a 'state of the science' review. Neuro Oncol 2014; 16: 896–913.

43   Weller M, Felsberg J, Hartmann C, Berger H, Steinbach JP, Schramm J et al. Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: a prospective translational study of the German Glioma Network. J Clin Oncol 2009; 27: 5743–50.

44   Molinaro AM, Wrensch MR, Jenkins RB, Eckel-Passow JE. Statistical considerations on prognostic models for glioma. Neuro Oncol 2016; 18: 609–23.

45   Walfish S. A review of statistical outlier methods. Pharm Technol 2006; 30: 82–88.

46   Noushmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, Berman BP et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. Cancer Cell 2010; 17: 510–22.

47   Cooper LAD, Gutman DA, Long Q, Johnson BA, Cholleti SR, Kurc T et al. The proneural molecular signature is enriched in oligodendrogliomas and predicts improved survival among diffuse gliomas. PLoS One 2010; 5: e12548.

48   Brennan CW, Verhaak RGW, McKenna A, Campos B, Noushmehr H, Salama SR et al. The somatic genomic landscape of glioblastoma. Cell 2013; 155: 462–77.

49   Piao Y, Liang J, Holmes L, Henry V, Sulman E, de Groot JF. Acquired resistance to anti-VEGF therapy in glioblastoma is associated with a mesenchymal transition. Clin Cancer Res 2013; 19: 4392–403.

50   Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD et al. Molecular

subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 2006; 9: 157–73.

51   Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W et al. IDH1 and IDH2 mutations in gliomas. N Engl J Med 2009; 360: 765–73.

52   Mahankali M, Farkaly T, Bedi S, Hostetler HA, Gomez-Cambronero J. Phosphatidic Acid (PA) can Displace PPARα/LXRα Binding to The EGFR Promoter Causing its Transrepression in Luminal Cancer Cells. Sci Rep 2015; 5: 15379.

53   Shinojima N, Tada K, Shiraishi S, Kamiryo T, Kochi M, Nakamura H et al. Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme. Cancer Res 2003; 63: 6962–70.

54   Hobbs J, Nikiforova MN, Fardo DW, Bortoluzzi S, Cieply K, Hamilton RL et al. Paradoxical relationship between the degree of EGFR amplification and outcome in glioblastomas. Am J Surg Pathol 2012; 36: 1186–93.

55   Faulkner C, Palmer A, Williams H, Wragg C, Haynes HR, White P et al. EGFR and EGFRvIII analysis in glioblastoma as therapeutic biomarkers. Br J Neurosurg 2014; 20:1-7.

56   Gangemi RMR, Daga A, Muzio L, Marubbi D, Cocozza S, Perera M et al. Effects of Emx2 inactivation on the gene expression profile of neural precursors. Eur J Neurosci 2006; 23: 325–34.

57   Falcone C, Daga A, Leanza G, Mallamaci A. Emx2 as a novel tool to suppress glioblastoma. Oncotarget 2016; May 13. [Epub ahead of print]

58   Wong J, Duncan CE, Beveridge NJ, Webster MJ, Cairns MJ, Weickert CS. Expression of NPAS3 in the human cortex and evidence of its posttranscriptional regulation by miR-17 during development, with implications for schizophrenia.

Schizophr Bull 2013; 39: 396–406.

59   Gould P, Kamnasaran D. Immunohistochemical analyses of NPAS3 expression in the developing human fetal brain. Anat Histol Embryol 2011; 40: 196–203.

60   Moreira F, Kiehl T-R, So K, Ajeawung NF, Honculada C, Gould P et al. NPAS3 demonstrates features of a tumor suppressive role in driving the progression of Astrocytomas. Am J Pathol 2011; 179: 462–76.

61   Palani M, Arunkumar R, Vanisree AJ. Methylation and expression patterns of tropomyosin-related kinase genes in different grades of glioma. Neuromolecular Med 2014; 16: 529–39.

62   Ding T, Zhou Y, Sun K, Jiang W, Li W, Liu X et al. Knockdown a water channel protein, aquaporin-4, induced glioblastoma cell apoptosis. PLoS One 2013; 8: e66751.

63   Ding T, Ma Y, Li W, Liu X, Ying G, Fu L et al. Role of aquaporin-4 in the regulation of migration and invasion of human glioma cells. Int J Oncol 2011; 38: 1521–31.