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 HR¹, White P², Hares KM³, Redondo J³, Kemp KC³, Singleton WGB⁴, Killick-Cole CL⁴, Stevens JR⁵, Garadi K⁶, Guglani S⁷, Wilkins A³, Kurian KM¹

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 PPAR\(\alpha\) 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,\(^8,9\) PPAR\(\alpha\) 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 PPAR\(\alpha\) has been reported to predict reduced clinical survival in ovarian carcinoma cohorts.\(^13\)

The role of PPAR\(\alpha\) 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 \textit{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 \textit{in vitro} models.
PPARα is overexpressed in HGG in vitro compared to normal human astrocytes and the expression of PPARα correlates with grade of malignancy in glial tumours. An association between hypoxic in vitro conditions and increased PPARα expression has been reported. 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. 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.\textsuperscript{26} Primary antibodies were PPAR\textalpha (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 Biosystems) and assay on demand (AoD) gene expression products for PPARα (Hs00947536_m1) (amplicon length 62bp) and GAPDH (Hs02758991_g1) (amplicon length 93bp) (Taqman® MGB probes, FAM dye-labelled, Applied Biosystems). The efficiency and linear range for both AoD were determined using a dilution series standard curve prior to expression analysis of the cohort. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

**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 and IDH1 R132H immunohistochemistry was performed.

**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. 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. The data was accessed and processed via the GlioVis online platform (https://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. Pearson correlation analysis was applied to significantly differentially expressed genes. Cross-validation with the Rembrandt glioblastoma data set 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
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 61 years. 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 59.7 years. 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 PPARα 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 PPARα expression. Differential gene expression analysis revealed gene subsets clustering with high (n=39) and low (n=31) PPARα expression (Figure 6). Of the gene expression values clustered with high PPARα expression, 10 have previously been associated with primary glioblastoma in published studies. Each of these 10 genes was significantly positively correlated with PPARα 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) (Table 7, Figure 7).

**DISCUSSION**

In this study we examined the expression of PPARα in IDH-wildtype glioblastoma, defined clinically as primary glioblastoma. 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.
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,\textsuperscript{39,40} is essential for on-going tumour progression or contributes to adjuvant therapy response,\textsuperscript{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 \textit{in vitro}.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 PPAR\(\alpha\) signalling has been associated with a variety of malignancies, the precise role of neoplastic PPAR\(\alpha\) 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 PPAR\(\alpha\) 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 PPAR\(\alpha\) 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 and its knockdown induces aggressive anaplastic astrocytomas in xenograft models. Demonstrated herein, NPAS3 expression additionally correlates with PPARα, 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. Conversely, AQP4 has been associated with anti-apoptotic and pro-invasive effects. Further work is required to determine the role of each of these molecular markers in the high PPARα 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 PPARα 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.
### Table 1: IHC scoring system for PPARα expression.

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

<table>
<thead>
<tr>
<th>Intensity</th>
<th>Score</th>
<th>Percentage Section</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO STAIN</td>
<td>0</td>
<td>NO STAIN</td>
<td>0</td>
</tr>
<tr>
<td>FAINT</td>
<td>1</td>
<td>1-25%</td>
<td>1</td>
</tr>
<tr>
<td>MODERATE</td>
<td>2</td>
<td>26-50%</td>
<td>2</td>
</tr>
<tr>
<td>STONG</td>
<td>3</td>
<td>51-75%</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>76-100%</td>
<td>4</td>
</tr>
<tr>
<td>Clinicopathological variable</td>
<td>Patients (n=100)</td>
<td>PPARα high</td>
<td>PPARα low</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-----------------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>68</td>
<td>40</td>
<td>28</td>
</tr>
<tr>
<td>Female</td>
<td>32</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;61</td>
<td>52</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
<td>≥61</td>
<td>48</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>KPS *</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥80</td>
<td>65</td>
<td>42</td>
<td>23</td>
</tr>
<tr>
<td>&lt;80</td>
<td>18</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>Tumour Location **</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal</td>
<td>25</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Non-frontal/diffuse/multifocal</td>
<td>67</td>
<td>42</td>
<td>25</td>
</tr>
<tr>
<td>MGMT promoter methylation ***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>49</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>49</td>
<td>32</td>
<td>17</td>
</tr>
</tbody>
</table>

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
**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
† 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

<table>
<thead>
<tr>
<th>Factor</th>
<th>n (events)</th>
<th>Median OS (months) [95% CI]</th>
<th>p-value (log rank)</th>
<th>Hazard Ratio [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>68 (53)</td>
<td>12.0 [10.64, 13.36]</td>
<td>0.149</td>
<td>1</td>
</tr>
<tr>
<td>Female</td>
<td>32 (34)</td>
<td>10.0 [4.04, 15.96]</td>
<td>1.36 [0.88, 2.09]</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28 – 55</td>
<td>32 (29)</td>
<td>13.0 [10.74, 15.27]</td>
<td>0.233</td>
<td>1</td>
</tr>
<tr>
<td>56 – 65</td>
<td>32 (27)</td>
<td>11.0 [7.67, 14.33]</td>
<td>1.43 [0.85, 2.42]</td>
<td></td>
</tr>
<tr>
<td>66 – 83</td>
<td>36 (31)</td>
<td>11.0 [7.08, 14.92]</td>
<td>1.48 [0.89, 2.46]</td>
<td></td>
</tr>
<tr>
<td>**KPS *</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤80</td>
<td>33 (29)</td>
<td>12.0 [5.25, 18.75]</td>
<td>0.429</td>
<td>1</td>
</tr>
<tr>
<td>81 – 90</td>
<td>33 (25)</td>
<td>12.0 [10.75, 13.25]</td>
<td>0.82 [0.48, 1.39]</td>
<td></td>
</tr>
<tr>
<td>91 - 100</td>
<td>23 (18)</td>
<td>15.0 [13.45, 16.55]</td>
<td>0.69 [0.38, 1.25]</td>
<td></td>
</tr>
<tr>
<td>**Localisation **</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal</td>
<td>25 (19)</td>
<td>13.0 [1.58, 24.42]</td>
<td>0.003</td>
<td>1</td>
</tr>
<tr>
<td>Non-frontal</td>
<td>61 (49)</td>
<td>13.0 [11.09, 14.91]</td>
<td>1.03 [0.60, 1.75]</td>
<td></td>
</tr>
<tr>
<td>Diffuse*</td>
<td>6 (6)</td>
<td>4.0 [0.00, 8.80]</td>
<td>4.07 [1.57, 10.59]</td>
<td></td>
</tr>
<tr>
<td>**Resection ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial</td>
<td>53 (44)</td>
<td>11.0 [6.54, 15.46]</td>
<td>0.041</td>
<td>1</td>
</tr>
<tr>
<td>Complete</td>
<td>39 (29)</td>
<td>14.0 [11.38, 16.62]</td>
<td>0.62 [0.39, 0.99]</td>
<td></td>
</tr>
<tr>
<td><strong>MGMT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>49 (37)</td>
<td>13.0 [9.08, 16.92]</td>
<td>0.171</td>
<td>1</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>49 (43)</td>
<td>12.0 [10.05, 13.95]</td>
<td>1.35 [0.87, 2.11]</td>
<td></td>
</tr>
<tr>
<td>**Treatment ****</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>† RT + TMZ full</td>
<td>39 (24)</td>
<td>24.0 [4.64, 43.36]</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>¥ RT + TMZ partial</td>
<td>24 (24)</td>
<td>9.0 [7.21, 10.79]</td>
<td>3.89 [2.12, 7.13]</td>
<td></td>
</tr>
<tr>
<td>RT alone</td>
<td>4 (4)</td>
<td>1.0 [- ----]</td>
<td>24.43 [7.24, 82.40]</td>
<td></td>
</tr>
<tr>
<td>Palliative</td>
<td>11 (10)</td>
<td>2.0 [- ----]</td>
<td>7.13 [3.31, 15.38]</td>
<td></td>
</tr>
<tr>
<td><strong>PPARα IHC</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.596</td>
</tr>
<tr>
<td>High</td>
<td>60 (46)</td>
<td>12.0 [9.13, 14.87]</td>
<td>0.596</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>40 (35)</td>
<td>12.0 [9.05, 14.95]</td>
<td></td>
<td>0.89 [0.57, 1.39]</td>
</tr>
</tbody>
</table>
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
<table>
<thead>
<tr>
<th>Factor</th>
<th>n</th>
<th>Median OS (months) [95% CI]</th>
<th>p-value (log-rank)</th>
<th>Hazard Ratio [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 – 55</td>
<td>161</td>
<td>14.7 [12.96, 16.49]</td>
<td><strong>&lt;0.001</strong></td>
<td>1</td>
</tr>
<tr>
<td>56 – 65</td>
<td>140</td>
<td>12.9 [10.91, 14.98]</td>
<td>1.36 [1.08, 1.72]</td>
<td></td>
</tr>
<tr>
<td>66 – 83</td>
<td>172</td>
<td>7.5 [5.50, 9.50]</td>
<td>2.27 [1.82, 2.85]</td>
<td></td>
</tr>
<tr>
<td><em>MGMT</em></td>
<td></td>
<td></td>
<td><strong>0.003</strong></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>141</td>
<td>12.2 [9.72, 14.68]</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Unmethylated</td>
<td>164</td>
<td>10.2 [8.75, 11.65]</td>
<td>1.42 [1.13, 1.79]</td>
<td></td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
<td><strong>&lt;0.001</strong></td>
<td></td>
</tr>
<tr>
<td>† RT + TMZ full</td>
<td>193</td>
<td>13.8 [12.64, 14.96]</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>¥ RT + TMZ partial</td>
<td>118</td>
<td>14.9 [13.18, 16.62]</td>
<td>0.74 [0.59, 0.94]</td>
<td></td>
</tr>
<tr>
<td>RT alone</td>
<td>135</td>
<td>5.4 [3.96, 6.84]</td>
<td>2.16 [1.73, 2.70]</td>
<td></td>
</tr>
<tr>
<td>Palliative</td>
<td>10</td>
<td>3.6 [1.43, 5.77]</td>
<td>3.70 [1.95, 7.03]</td>
<td></td>
</tr>
</tbody>
</table>

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.

† 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
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.

\(^a\) Includes full 60Gy (30) plus concurrent TMZ with full 6 cycles adjuvant TMZ

\(^b\) 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

\(\dagger\) 17 missing data points
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.

<table>
<thead>
<tr>
<th>PPARA vs gene</th>
<th>TCGA dataset, Pearson’s r (95% CI)</th>
<th>Rembrant dataset, Pearson’s r (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>0.24 (0.15-0.32)</td>
<td>0.37 (0.25-0.48)</td>
</tr>
<tr>
<td>EMX2</td>
<td>0.28 (0.20-0.36)</td>
<td>0.28 (0.15-0.40)</td>
</tr>
<tr>
<td>AQP4</td>
<td>0.27 (0.19-0.35)</td>
<td>0.34 (0.21-0.45)</td>
</tr>
<tr>
<td>NPAS3</td>
<td>0.35 (0.27-0.42)</td>
<td>0.21 (0.08-0.33)</td>
</tr>
<tr>
<td>NTRK2</td>
<td>0.30 (0.21-0.38)</td>
<td>0.30 (0.21-0.38)</td>
</tr>
</tbody>
</table>
Acknowledgements:

The Pathological Society and Jean Shanks Foundation Pathological Research Training Fellowship (H.R.H). The Brain Tumour Bank South West (BRASH) at North Bristol NHS Trust UK. FFPE Tissue samples were obtained from North Bristol NHS Trust as part of the UK Brain Archive Information Network (BRAIN UK) which is funded by the Medical Research Council and Braintrust. W.G.B.S. is a Medical Research Council Clinical Research Training Fellow joint funded between the Medical Research Council and The Brain Tumour Charity. The results published here are in part based upon data generated by the TCGA Research Network: (http://cancergenome.nih.gov/). The authors wish to thank Drs Sean Elyan and Lara Gibbs plus The National Cancer Registration and Analysis Service (part of Public Health England) for their assistance with the clinical data acquisition.

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


6 Ellis HP, Kurian KM. Biological Rationale for the Use of PPARγ Agonists in Glioblastoma. Front Oncol 2014; 4: 52.


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.


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


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.


