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An evaluation of the quality of RNA extracted from archival FFPE glioblastoma and epilepsy surgical samples for gene expression assays

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ABSTRACT (247 words)

Aims: Histopathological tissue samples are being increasingly utilised as sources of nucleic acids in molecular pathology translational research. This study investigated the suitability of glioblastoma and control CNS formalin fixed paraffin embedded (FFPE) tissue-derived RNA for gene expression analyses.

Methods: Total RNA was extracted from control (temporal lobe resection tissue) and glioblastoma FFPE tissue samples. RNA purity (260/280 ratios) was determined and RNA integrity number (RIN) analysis performed. RNA was subsequently used for RT-qPCR for two reference genes, *18S* and *GAPDH*.

Results: Reference gene expression was equivalent between control and glioblastoma tissue when using RNA extracted from FFPE tissue, which has key implications for biological normalisation for CNS gene expression studies. There was a significant difference between the mean RIN values of control and glioblastoma FFPE tissue. There was no significant correlation between 260/280 or RIN values vs total RNA yield. The age of the tissue blocks did not influence RNA yield, fragmentation or purity. There was no significant correlation between RIN or 260/280 ratios and mean cycle threshold (C_t) for either reference gene.

Conclusions: This study showed that routinely available CNS FFPE tissue is suitable for RNA extraction and downstream gene expression studies, even after 60 months of storage. Substantial RNA fragmentation associated with glioblastoma and control FFPE tissue blocks did not preclude downstream RT-qPCR gene expression analyses. Cross validation with both archival and prospectively collated FFPE specimens is required to further demonstrate that CNS tissue blocks can be utilised in novel translational molecular biomarker studies.

INTRODUCTION

Gliomas are the most common primary CNS tumours, with an incidence of 6.6 per 100,000 individuals per year [1]. Approximately 50% of newly diagnosed gliomas are glioblastomas [2]. The median patient survival is approximately 15 months in the setting of a clinical trial [3,4] and only 12 months using the current established treatment regimens [1,5].

Histopathological diagnosis of glioblastoma is dependent on formalin-fixed paraffin embedded (FFPE) tissue obtained from a biopsy or surgical resection. Despite a number of practical issues regarding using this diagnostic tissue for research, the number of molecular studies incorporating FFPE material is increasing [6].

The high quality morphological information that FFPE tissue provides facilitates accurate macro- and microdissection of areas of interest. This means that with downstream nucleic acid purification and enrichment, histopathological abnormalities (for example the malignant transformation of a cellular population or subpopulation), can be interrogated using molecular tools [7]. This approach has been widely used in the discovery phase of biomarker investigation for large-scale validation and for subsequent implementation into routine clinical practice [8].

Tumour tissue is a heterogeneous milieu of non-transformed cells, immune cells, stromal cells and areas of necrosis in which the neoplastic cells may only represent a small proportion. Therefore, accurate assessment of tumour cell composition by expert histopathologists based upon morphological features is essential. This assessment can then guide nucleic acid extraction for subsequent molecular analyses. This may be done manually, using pre-marked haematoxylin and eosin (H&E) sections to guide dissecting areas of tissue (i.e. macrodissection) or using automated laser capture microdissection [9], with the emerging possibility of single cell analysis [10]. Techniques such as expression microdissection may allow for enrichment of extracted cell populations by immunohistochemical labelling [11]. In contrast,

the use of fresh frozen (unfixed) tissue is associated with the homogenisation of tissue samples (for downstream protein, DNA or RNA extraction), a bulk analysis approach in which the input cellular material may be sub-optimally (or blindly) selected.

RNA isolated from FFPE tissue is often of lower quality than that obtained from fresh frozen tissue, frequently showing evidence of degradation, fragmentation and reduced assay efficiency [12]. Nucleic acid quantity and quality may also be dependent on the extraction method utilised [13,14]. It is therefore important that total RNA extracted from FFPE samples is adequately assessed before being assayed in downstream molecular applications.

This study examined the applicability of FFPE tissue for RNA extraction for subsequent molecular expression analyses in the context of glioblastoma. Quality control (QC) metrics were assessed for FFPE tissue-derived RNA for glioblastoma and (control) epilepsy resection specimens.

MATERIALS AND METHODS

Study samples

IDH1[R132H]-wildtype (primary) glioblastoma FFPE surgical specimens [15], diagnosed according to WHO diagnostic criteria were obtained from the Brain Tumour Bank Southwest, UK. FFPE tissue samples from histologically normal anterior temporal lobe surgical resections were used as controls. Control tissue samples were provided under *BrainUK* ethical approval (14/008, 15/017).

Tumour FFPE sample area selection for macrodissection

Two histopathologist researchers (HRH & KMK) reviewed H&E stained FFPE glioblastoma sections and marked areas containing only tumour cells. Any areas containing extensive tissue necrosis, haemorrhage, microvascular proliferation or histologically normal cerebral cortex were marked for exclusion from downstream total RNA extraction.

RNA extraction from FFPE tissue sections

Tissue blocks were sectioned at 5μm and stored in the absence of light at ambient temperature for a maximum of 21 days. Total RNA was extracted from either whole control tissue (n=17; x10 5μm FFPE unstained sections) or macrodissected regions of glioblastoma tissue (n=48; x10 5μm FFPE unstained sections). Total RNA was extracted using an EZNA FFPE RNA kit (OmegaBio-tek) including a gDNA elimination spin column step, as previously described [16]. Purified total RNA was eluted into RNase-free water. RNA was stored at -80°C until use.

RNA Quality and Quantity

RNA concentration and 260/280 ratios were determined using a Nanodrop 1000 UV spectrophotometer (ThermoScientific). RNA integrity number (RIN) analysis was performed using an Agilent 2100 Bioanalyser and RNA 6000 LabChip kit with Agilent 2100 Expert software (Agilent Technologies). 260/280 ratios and RIN were determined for all control

samples (n=17) and a subset of glioblastoma samples (selected randomly n=29). Two RIN values were not detected using the Agilent 2100 Instrument in the glioblastoma cohort. Total RNA was reverse transcribed to cDNA using a Clontech TaKaRa PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa Bio) following the manufacturer's protocol.

Linear dynamic range (LDR) and efficiency of RT-qPCR reactions

The linear dynamic range (LDR) for each Taqman® assay on demand (AOD) was investigated prior to gene expression analyses. Taqman® fast gene expression mastermix (Applied Biosystems) was used. The LDR of each AOD was assessed using a standard curve method, utilising serial dilutions of cDNA template. The log of cDNA starting quantity against the cycle threshold (C_t) value obtained during amplification was plotted for each dilution. Experiments were performed in triplicate using the StepOnePlus Real-Time PCR system and StepOne software v2.1 (Applied Biosystems), which provided an automated output to fit the equation of the linear regression line and the coefficient of determination (R^2). Two representative cDNA samples from each of the control and glioblastoma subsets of the FFPE cohort were used for this optimisation.

Reverse transcription quantitative real-time PCR (RT-qPCR)

RT-qPCR was performed with a StepOnePlus instrument with Taqman Fast Gene Expression Mastermix (both Applied Biosytems) and AOD gene expression products for *GAPDH* (Hs02758991_g1) (amplicon length, 93 bp) and *18S* (Hs03003631_g1) (amplicon length, 69 bp) (Taqman MGB probes, FAM dye-labelled; Applied Biosytems). Experiments were performed in triplicate. Relative gene expression was analysed with the $2^{-\Delta\Delta Ct}$ method [17] and the geomean calculated for each group. No-template control (1μl nuclease-free water) and RT- (gDNA contamination) control samples were included in each PCR run. The amplification baseline and threshold were automatically calculated by the StepOne software.

Statistical Analysis

Data normality was tested using the Kolmogorov-Smirnov and the D'Agostino and Pearson omnibus normality tests. A Mann-Whitney test or unpaired *t*-test was used to compare the parameters RIN, age of block and 260/280 ratios in control versus disease tissue. Correlation analysis was performed using Pearson correlation coefficient (parametric data) or Spearman rank correlation (non-parametric data) as appropriate. All statistical tests were two-tailed. Differences at $p < 0.05$ were considered statistically significant. Statistical tests were performed using GraphPad Prism v5 for Windows (GraphPad).

RESULTS

The linear dynamic range (LDR) of RT-qPCR AOD

AOD optimisation was performed for the reference genes *GAPDH* and *18S* by generating a standard curve prior to downstream quantification of relative gene expression. The R^2 for both *GAPDH* and *18S* was >0.980 (Figure 1a&b), indicative of a strong linear relationship between absolute starting template and assay output. Downstream relative gene expression studies were performed using RT-qPCR and starting cDNA concentrations corresponding to the median cDNA concentration within the LDR for each AOD.

Reference gene expression in control versus glioblastoma FFPE tissue

RT-qPCR was performed on cDNA generated from FFPE control and glioblastoma tissue blocks. There was no significant difference in the expression of the reference gene *GAPDH* in the glioblastoma samples compared to control samples when normalised to *18S* mRNA expression (p=0.576; Figure 2a). Similarly, there was no significant difference in the expression of the reference gene *18S* in glioblastoma samples compared to control samples, when normalised to *GAPDH* (p=0.575; Figure 2b).

Analysis of RNA quality control metrics in control and glioblastoma FFPE tissue samples

The mean RIN in the control FFPE tissue cohort was 2.2 (range: 1.3-2.7). The mean RIN in the glioblastoma FFPE tissue cohort was 1.7 (range: 1.0-2.7). There was a significant difference in the RIN value between these 2 groups (p=0.008) (Figure 3a). The mean age of the blocks (months) in the control cohort was 24.9 (range: 3-60). The mean age of the blocks (months) in the glioblastoma cohort was 32.9 (range: 14-55). There was no significant difference in the age of the blocks between these 2 groups (p=0.097) (Figure 3b). The mean 260/280 ratio in the control cohort was 1.94 (range: 1.63-2.05). The mean 260/280 ratio in the glioblastoma cohort was 1.95 (range: 1.63-2.26). There was no significant difference in the 260/280 ratio between these 2 groups (p=0.715) (Figure 3c). The mean percentage tumour (by area of total tissue per slide) in the glioblastoma FFPE tissue cohort alone was 61% (range: 15-100%).

There was no statistically significant correlation between total RNA yield (ng/µl) vs RIN in the control and glioblastoma FFPE tissue blocks as a single group (p=0.250) (Figure 4a). Furthermore, there was no statistically significant correlation between total RNA yield (ng/µl) vs 260/280 ratio in the control and glioblastoma FFPE tissue blocks as a single group (p=0.172) (Figure 4b).

There was no significant correlation between age of the block vs RIN in the control or glioblastoma FFPE tissue blocks (p=0.249, p=0.983 respectively) (Figure 5a&b) and no significant correlation between age of the block vs total RNA yield (ng/µl) in the control or glioblastoma FFPE tissue blocks (p=0.268, p=0.252; respectively) (Figure 5c&d); although a non-significant overall negative trend was noted, particularly for the glioblastoma FFPE tissue blocks. There was no significant correlation between age of the block vs 260/280 ratios in the control or glioblastoma FFPE tissue blocks (p=0.384, p=0.133 respectively) (Figure 6a&b).

There was no significant correlation between RIN vs (raw) mean C_t values for *GAPDH* or *18S* mRNA expression values in the control and glioblastoma FFPE tissue blocks combined (p=0.119, p=0.057 respectively) (Figure 7a&b), although a non-significant overall negative trend was noted, particularly for mean *18S* mRNA expression values (Figure 7b). There was a statistically significant negative correlation between 260/280 ratios vs (raw) mean C_t values for *GAPDH* and *18S* mRNA expression values in the control and glioblastoma FFPE tissue blocks combined (p=0.013, p=0.006 respectively) (Figure 7c&d).

DISCUSSION

It is essential that new tools are developed that better delineate the biological variants of glioblastoma. However, to date molecular assays that determine which newly diagnosed glioblastoma patients may respond to standard or experimental adjuvant therapies have proven elusive. Without a more finessed approach, treatment targets may be missed and patients given toxic therapies not sufficiently targeted to their tumour subtype [18]. In addition, molecular profiling can facilitate the identification of personalised prognostic features which can be used for patient stratification in clinical trial design, to ensure balance in the arms of randomised control trials of novel glioblastoma therapies [19].

Although data from targeted gene panel massive parallel sequencing approaches are already used to guide clinical decision making at molecular tumour boards [20], RNA expression analysis is likely to continue to have significant utility, complementing high-throughput sequencing of gDNA [21,22]. Gene expression studies have traditionally been carried out on nucleic acids extracted from fresh (snap) frozen tissue. More recently however FFPE tissue is being increasingly utilised due to its abundance in clinical diagnostic pathology archives and a growing interest in how to best control for the limitations inherent in FFPE based genetic studies [23–25]. For example, the Onco*type* DX® clinical assay, which uses FFPE-derived RNA

in RT-qPCR applications, is routinely used to guide adjuvant treatment decisions for breast cancer patients [26]. This use of RNA expression analysis adds predictive value to established clinicopathological and protein expression variables and is applicable to routinely available FFPE specimens. Although similar approaches have been investigated for glioblastoma [27,28], to date none have been extensively cross validated or achieved widespread acceptance. In order for this powerful approach to be more widely implemented, a better understanding of the limiting factors in the molecular investigation of FFPE glioblastoma (and control) tissue-derived nucleic acids is required.

In this study, the quality and quantity of RNA extracted from glioblastoma and control FFPE tissue was examined. Normalisation of RT-qPCR data to *GAPDH* or *18S* is commonly used in gene expression studies, as their expression is considered to be ubiquitous in the entire cell population present in a tissue sample. This study demonstrated that there was no significant difference between *GAPDH* and *18S* mRNA expression between control and glioblastoma samples, strongly suggesting that their expression is consistent between the two tissue cohorts. This is an important consideration when selecting reference genes for biological normalisation to perform quantitative mRNA studies comparing CNS tissue cohorts [29,30] and requires further cross validation with independent tissue sources.

During the optimisation of AOD used for downstream RT-qPCR analysis, *GAPDH* and *18S* reference genes both returned high regression coefficient variables, suggesting low variability between technical replicates and indicating that the PCR amplification efficiency was the same regardless of the starting template copy number. This is appropriate for studies on RNA extracted from FFPE tissue where the starting template amount may vary.

In this study, a single RNA FFPE extraction kit and protocol was used to extract total RNA from both primary glioblastoma and histologically normal anterior temporal lobe surgical resection samples derived from a single diagnostic archive. QC metrics were applied to the extracted total RNA prior to reverse transcription. Glioblastoma surgical samples that were

subjected to RIN analysis had significantly lower RIN values compared to control tissue. It is likely that the lower RIN values obtained for the glioblastoma cohort occurred due to increased levels of apoptosis and necrosis occurring in the tumour tissue. Although substantial RNA degradation was noted, the mean RIN and RIN range was comparable to that found in previously published studies which utilised RNA extracted from FFPE tissue samples, both for human glioma samples [31,32] and other common cancers [13,33]. It is increasingly recognised that low RIN values (i.e. 1.0) do not preclude successful outcomes in gene expression studies [34,35].

The RIN and 260/280 ratios, pooled across both control and glioblastoma FFPE tissue blocks, showed no significant correlation with total RNA yield, suggesting that neither fragmentation of extracted RNA nor its purity impacted on downstream quantity measurements. This is in keeping with previous reports which suggest that the quality and quantity of RNA extracted from FFPE tissues are likely to be independent factors: RNA quality being affected primarily by preanalytical factors and RNA quantity by the extraction technique employed [36]. This effect has also been reported in snap-frozen CNS tissue (non-neoplastic) cohorts [37].

In both control and glioblastoma tissue, RIN values did not correlate significantly with the age of the blocks, a finding in keeping with previously published work on FFPE breast carcinoma samples [33]. Similarly, both total RNA yield and 260/280 ratios were not found to be correlated with the age of the tissue blocks. This has important implications for future investigations which may utilise retrospective FFPE tissue cohorts for translational molecular studies in which longer clinical follow up is required.

There was no significant correlation between RIN values and raw C_t values obtained for the reference genes *GAPDH* and *18S*, in keeping with previous findings [38]. It is noteworthy that 260/280 ratios did, however, show a negative correlation with raw *GAPDH* and *18S* C_t values. A 260/280 ratio of 2.0 is widely accepted as "pure" for RNA samples [39]: the lower values noted in this study are likely to be due to contamination by materials present in the FFPE sections or reagents utilised during RNA extraction from the FFPE tissue. The increasing raw C_t values

noted to correlate with the lower 260/280 ratios may have occurred due to a decrease in PCR amplification efficiency by inhibitory contaminants. This effect is predicted to occur for both any putative genes of interest and the reference genes described herein and is therefore less likely to influence downstream relative gene expression outputs.

In summary, this study showed that routinely available FFPE CNS tissue samples are suitable for RNA extraction and downstream gene expression studies, even after 60 months of storage. In addition, it was shown that substantial RNA fragmentation associated with glioblastoma and control cortex FFPE tissue blocks does not preclude successful downstream RT-qPCR gene expression analyses. Cross validation with both archival and prospectively collated FFPE specimens is required to further demonstrate that CNS tissue blocks can be utilised in novel translational molecular biomarker studies.

Key messages:

1.Careful quality control of nucleic acids extracted from FFPE tissues is key to successful molecular analyses.

2. Prolonged FFPE tissue storage may not affect the quality or quality of RNA extracted.

3.Substantial RNA fragmentation associated with FFPE tissue does not influence quantitative outputs in appropriately designed RT-qPCR analyses.

Author contributions

Study conception and design: H. R. Haynes, K. M. Hares, K. C. Kemp, C. Faulkner. Data collection, analysis and interpretation: H. R. Haynes, C.L Killick-Cole, K. M. Hares, K. C. Kemp, J Redondo, C. Faulkner. Manuscript production: H. R. Haynes, C. L. Killick-Cole, K.A Moutasim, A. Wilkins. Final approval of manuscript: H. R. Haynes, A. Wilkins, and K. M. Kurian.

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Competing Interest:

None declared.

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Figure 1: Representative amplification plot and standard curve.
Six-fold dilutions of cDNA were used to optimise assays on demand (AOD). **(A)** Upper panel shows the *GAPDH* amplification plot, with **(B)** Corresponding standard curve in the lower panel. Standard curves were constructed for each AOD by plotting raw Ct values vs log (input cDNA). Similar results were obtained for *18S.*

Figure 2: Reference gene expression by RT-qPCR using FFPE extracted RNA
A) *GAPDH* mRNA expression was examined in FFPE samples of control cortex (n = 17) and glioblastoma (n = 48) by RT-qPCR, normalised to the reference gene *18S.* **(B)** *18S* mRNA expression was examined in FFPE samples of control cortex (n = 17) and glioblastoma (n = 48) by RT-qPCR, normalised to the reference gene *GAPDH.* The geometric mean and 95% confidence interval are shown on a logarithmic scale (to base2). The test statistic is a Mann–Whitney test two-tailed p-value. ns, non-significant. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *18S*, 18S ribosomal RNA.

Figure 3: RIN values, age of FFPE tissue blocks and RNA 260/280 values: control vs glioblastoma FFPE samples.
(A) RIN values for RNA extracted from FFPE control (n=17) vs glioblastoma (n=27, 2 missing RIN values) samples. **(B)** Age of the tissue block (months) used for RNA extraction, control (n=17) vs glioblastoma (n=29) samples. **(C)** 260/280 ratios for RNA extracted from FFPE control (n=17) vs glioblastoma (n=29) samples. The test statistic is a Mann–Whitney test two-tailed p-value (A and C) or unpaired t-test two-tailed p-value (B). Error bars show SEM. ns, non-significant; ** p<0.01.

Figure 4: **RIN values vs total RNA yield and 260/280 ratios vs total RNA yield**
(A) RIN values for RNA extracted from FFPE samples vs total RNA yield (n=44, 2 missing RIN values). **(B)** 260/280 ratios for RNA extracted from FFPE samples vs total RNA yield (n=46). r_s; Spearman's rank (nonparametric) correlation coefficient. Dotted lines; 95% CI. *x* axis shown on a logarithmic scale (to base2) (A&B).

Figure 5: RIN values and total RNA yield vs age of control and glioblastoma FFPE blocks.
(A) RIN values for RNA extracted from FFPE samples vs age of control FFPE blocks (months) (n=17). **(B)** RIN values for RNA extracted from FFPE samples vs age of glioblastoma FFPE blocks (months) (n=27, 2 missing RIN values). **(C)** Total RNA yield vs age of control FFPE blocks (months) (n=17). **(D)** Total RNA yield vs age of glioblastoma FFPE blocks (months) (n=29). r_s; Spearman's rank (nonparametric) correlation coefficient; Pearson *r*, Pearson (parametric) correlation coefficient. Dotted lines; 95% CI.

Figure 6: 260/280 ratios vs age of control and glioblastoma FFPE tissue blocks
(A) 260/280 values for RNA extracted from FFPE samples vs age of control FFPE blocks (months) (n=17). **(B)** 260/280 values for RNA extracted from FFPE samples vs age of glioblastoma FFPE blocks (months) (n=29). r_s; Spearman's rank (nonparametric) correlation coefficient. Dotted lines; 95% CI.

Figure 7: RIN values and 260/280 ratios vs (raw) mean Ct values
RIN values for RNA extracted from FFPE samples vs **(A)** *GAPDH* mean PCR C_t values (n=44) and **(B)** *18S* mean PCR C_t values (n=44). 260/280 ratios for RNA extracted from FFPE samples vs **(C)** *GAPDH* mean PCR C_t values (n=46) and **(D)** *18S* mean PCR C_t values (n=46). r_s; Spearman's rank (nonparametric) correlation coefficient; Pearson *r*, Pearson (parametric) correlation coefficient. Dotted lines; 95% CI.