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Validation of Reference Genes for Expression Studies in Human Meningiomas under Different Experimental Settings

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Abstract Quantitative polymerase chain reaction (qPCR) is a sensitive technique for the quantitative analysis of gene expression levels. To compare mRNA transcripts across tumour and non-pathological tissue, appropriate reference genes are required for internal standardisation. Validation of these reference genes in meningiomas has not yet been reported. After mRNA transcription of meningioma (WHO grade I-III) and meningeal tissue from three different experimental sample types (fresh tissue, primary cell cultures and FFPE tissue), 13 candidate reference genes (*ACTB*, *B2M*, *HPRT*, *VIM*, *GAPDH*, *YWHAZ*, *EIF4A2*, *MUC1*, *ATP5B*, *GNB2L*, *TUBB*, *CYC1*, *RPL13A*) were chosen for quantitative expression analysis. Two statistical algorithms (GeNorm and NormFinder) were used for validation of gene expression stability. All candidate housekeepers tested for stability were checked within and across the three tissue analysis groups. Pearson correlation, the ΔC_t method and ranking analysis identified the most non-regulated genes suitable for internal standardisation. *TUBB*, *HPRT* and *ACTB* were the most stably expressed genes for all analysis groups across meningioma and non-pathological meningeal tissue combined. In contrast, analysis of the consistency of reference gene expression within specific meningioma and meningeal tissues resulted in specific reference gene rankings for each tissue type. Future gene expression analyses require reference genes to be chosen that are suitable for the tissue types and for the experimental paradigms being studied. Validation of candidate housekeeper genes in meningiomas for quantitative real-time polymerase chain reaction revealed for the first time *TUBB*, *ACTB* and *HPRT* as the most consistently expressed genes among meningioma and non-pathological meningeal tissue across a range of experimental settings.

Keywords Meningioma · Meningeal neoplasms · Real-time polymerase chain reaction · Reference standards · Gene expression · Cell culture techniques

Introduction

Quantitative polymerase chain reaction (qPCR) is currently one of the most precise methods for studying the expression profiles of specific gene transcripts, which are differently expressed in meningiomas depending on their respective WHO grade. Using this technique, both the absolute and the relative amount of RNA can be quantified [1, 2]. With regard to relative quantification with qPCR, its precision and reliability depend on the appropriate choice of stable internal controls for normalisation [3]. In terms of qPCR-based analysis of gene expression profiles in meningiomas, normalisation of the results in relation to unregulated reference genes as an internal standard is mandatory [1, 4]. Therefore, it is common to normalise qPCR data against two or even three different, steadily expressed genes [4]. Optimal reference genes will be nearly constantly expressed in all tissues to be tested, while the use of inappropriate reference genes may lead to an impairment of detection sensitivity of the target genes, leading ultimately to incorrect results [5, 6]. The most frequently used reference

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gene for qPCR assays in general is glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) [7], although it is known that *GAPDH*, under specific conditions, can show inconsistent expression levels in comparison to controls [5, 8]. In autopsy brain tissue from patients with Alzheimer's disease, *RPL13A* and *18S* were identified as the most stably expressed genes and, as a consequence, the most suitable housekeeping gene candidates [5]. Further, frequently used reference genes include beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase 1 (*HPRT*) and beta-actin (*ACTB*) [9, 10].

Nevertheless, there is little reliable data on molecular markers and gene expression levels related to unregulated housekeeper genes in the setting of meningiomas.

Meningiomas account for up to 26% of all intracranial tumours and with an annual incidence of 6 per 100,000 people they are the most common tumours arising from the meninges [11, 12]. Histopathological grading of those tumours is based on the current classification of the World Health Organization (WHO), which divides them into three groups: classic/benign (WHO grade I), atypical (WHO grade II) and anaplastic (WHO grade III) [13]. The majority of meningiomas (~ 90%) are classified as classic, followed in incidence by atypical (5–7%) and anaplastic (1–3%) [14–16]. Little is known of the tumourigenesis, malignant progression and genetics of meningiomas, especially in comparison with other intracranial tumours such as gliomas. An early event in meningioma tumourigenesis might be mutation of the *NF2* gene on chromosome 22q12, which is seen in up to 60% of all sporadic meningiomas, presumably resulting in a loss of function of the merlin (or schwannomin) protein [17, 18]. In familial meningiomas, the product of the *DAL-1* gene on chromosome 18p11, which has a homology with merlin, has been implicated. In addition to numerous genetic deletions (22q, 1p, 3p, 6q, 9p, 10q, 14q) [19, 20], comparative genomic hybridisation studies have demonstrated allelic gain and amplification of 17q in up to 60% of anaplastic meningiomas [19]. Gene expression profiling has confirmed altered expression of growth hormone receptor (*GHR*), insulin-like growth factor II (*IGF II*) and the IGF-binding protein 7 as well as various gene overexpression affecting *EAR-2* and *cathepsin K*. In addition, downregulation of gene expression for *RAD*, *BCR*, and *JUN-B* [21] is seen with increasing WHO tumour grade, perhaps with implications for the malignant transformation of the tumours.

This study aimed to validate specific reference genes in meningiomas, testing their stable expression under a variety of treatment conditions and across different tissue samples. Furthermore, potential moderator variables such as the age and gender of the patients were taken into account due to their possible impact on reference gene expression.

There is a large panel of statistical calculation methods to identify the most stable expression among a set of candidate genes and each one refers to a different background to determine the stability of a gene. One of those is the NormFinder algorithm, which calculates the variation using a side-by-side comparison. A relevant disadvantage of this algorithm is its loss of sensitivity towards coregulation [3]. Another potential calculation model is the geNorm algorithm, which determines the most stable pair of genes by a stepwise exclusion of least stable candidates [22]. Further techniques to compare and rank the tested candidate reference genes include the ΔC_t method [23] and Pearson correlation analysis.

Materials and Methods

Tumour Samples

To validate the suitability of potential reference genes for qPCR normalisation, a total number of 19 human meningioma (WHO grade I $n = 9$, WHO grade II $n = 7$, WHO grade III $n = 3$) and mixed leptomeningeal ($n = 4$) ex vivo tissue samples, 20 ($n = 5$ for each WHO grade and dural tissue) formaldehyde-fixed, paraffin-embedded (FFPE) tissue samples and 14 classified primary cell culture samples (in vitro) ($n = 6$ originating from WHO grade I tumours, $n = 2$ from grade II tumours, $n = 2$ from grade III tumours, $n = 2$ dural cell cultures and $n = 2$ arachnoidal cell cultures) were investigated.

Surgical tissue was immediately stored at − 80 °C and made available for analysis by qPCR. The FFPE samples were provided by the Institute of Neuropathology, Charité - Universitätsmedizin Berlin. Primary cell cultures were compounded directly after surgical resection. All tissue samples were histopathologically classified based on the 2007 WHO classification of brain tumours [15]. The study was approved by the local human research ethics committee.

Total RNA Extraction, Quantification and Reverse Transcription

Total RNA was isolated from frozen tissue and cell culture samples lysed in Qiazol (Qiagen GmbH, Hilden, Germany) and purified with the RNeasy Mini Kit (Qiagen GmbH) according to the manufacturer's protocol. Isolation of total RNA from formalin-fixed, paraffin-embedded (FFPE) tissue was performed with the High Pure FFPE RNA Micro Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the instructions for use.

The RNA concentration was determined by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA).

Concentrations of least 10 ng/μl RNA were used for synthesis. Complementary DNA synthesis was performed using the GoScript Reverse Transcription System (Promega Corp., Mannheim, Germany) in a volume of 20 μl per reaction in six cycles according to the manufacturer's instructions.

Candidate Reference Genes

In this study we tested 13 different candidate genes for normalisation in meningiomas versus non-pathological dural tissue and arachnoid membrane (leptomeningeal samples). This subset of tested candidate genes represented several functional classes, which reduced the possibility of coregulation and false positive gene selection. We tested glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), beta-actin (*ACTB*), ribosomal protein L13a (*RPL13A*), cytochrome c-1 (*CYC1*), beta-2a-tubulin (*TUBB*), guanine nucleotide binding protein (*GNB2L*), mucin 1 (*MUC1*), eukaryotic translation initiation factor 4A2 (*EIF4A2*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (*YWHAZ*), vimentin (*VIM*), beta-2-microglobulin (*B2M*), hypoxanthine phosphoribosyltransferase 1 (*HPRT*), and ATP synthase beta polypeptide (*ATP5B*) as potential reference genes for classical, atypical and malignant meningiomas and non-pathological leptomeningeal tissue to determine the potential usefulness of these genes for normalisation in future functional studies (Table 1).

Quantitative PCR

As described previously, human-specific primers for qPCR, based on mRNA coding sequences (GenBank), were designed using NetPrimer (Premier BioSoft, Palo Alto, CA, USA; http://www.premierbiosoft.com/netprimer/) and NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; sequences listed in Table 1) and checked for hairpins or other secondary structures [24]. The specificity of the amplification was analysed by gel electrophoresis and melting curve analysis. Quantitative PCR (qPCR) was performed in a 20-µl amplification mixture containing 2× MasterMix (DyNamo Flash SYBR Green qPCR Kit, Thermo Fisher Scientific), specific primers, each in a final concentration of 500 nM, and cDNA (25 ng cDNA equivalent to reverse transcripted RNA). The specific transcripts were amplified in Rotor-Gene G (Qiagen GmbH) under the following conditions: 7-min polymerase activation, 40 amplification cycles (95 °C for 10 s, 55 °C for 20 s, 72 °C for 30 s).

Data Analysis

The expression profiles of 13 candidate genes were analysed based on the various statistical models in order to validate the quality of each of these preselected genes as the one most suitable for normalisation. The baseline subtracted threshold cycles (Corbett Rotor-Gene 6000 Software) from two intra-assay samples were averaged and analysed. Relative gene expression values (R), accounting for the different amplification efficiencies (E) for between treatment and control group were calculated according to Eq. (1). The efficiency was calculated with a linear regression of the C_t values of a standard dilution series (Eq. 2) [25].

$$R = E^{(Ct_{control} - Ct_{treatment})} \quad (1)$$

$$E = 10^{-(1/slope)} \quad (2)$$

To determine the relation between the 13 tested transcripts, their absolute expression was calculated in comparison to the remaining genes.

Statistical Analysis

The Ct values of each sample were calculated with the Microsoft Excel-based software applications NormFinder [3] and geNorm [22], and using the ΔC method [23] and Pearson correlation analysis. These algorithms determined the stability and the ranking of the reference genes following the method implemented by Cheng et al. [26].

Results

RNA Quality, Efficiency and Technical Viability

We assessed the quality of the RNA used as starting material in several ways. Optical density absorption ratios A_{260}/A_{280} were measured by NanoDrop 2000 (Thermo Fisher Scientific). RNA from cell cultures and tissue samples showed a mean ratio of 1.95 (±0.2 SD) and RNA isolated from FFPE tissue had a mean ratio of 1.57 (±0.4 SD), reflecting higher purity of the fresh versus the formalin-fixed tissues. Additionally, the purity and integrity of RNA was verified by denaturing formaldehyde agarose gel electrophoresis. All samples showed clear and specific bar for intact RNA and were used for further analysis.

The efficiency of gene amplification was calculated as described above, with results ranging from 1.8 to 2.3. All non-normalised baseline-subtracted threshold cycle (C_t) values across analysis groups were within the range of the dilution series. The determination coefficient ranged from 0.978 to 0.999.

Expression Levels of Candidate Genes

We found a spectrum of C_t values representing a wide difference in expression of the candidate genes, ranging between 12.46 and 27.08 for fresh frozen tissues, 13.92 and 33.89 for fresh cell culture samples, and 21.24 and 39.01 for measured gene expression in FFPE samples of mixed meningioma and dural tissues. Figure 1 illustrates the respective C_t values of each sample group for all 13 tested candidate reference genes

Table 1 Pre-selected candidate reference genes and their sequences, the specific fragment size, along with the function of the genes and their proteins

Gene (fragment size)	Forward/reverse primers (5′ sequences 3′)	Name	Reference	Function
ATP5B (159 bp)	cccttctgctgtgggctatc tgggcaaacgtagtagcagg	*Homo sapiens ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide*	NM_001686.3	Purine nucleotide/ adenosine ribonucleotide biosynthesis (metabolism)
B2M (238 bp)	catggaggtttgaagatgccgc ccccacctctaagttgccagcc	*Homo sapiens beta-2-microglobulin*	NM_004048.2	Component of MHC class I (immune system)
CYC1 (160 bp)	gaggtggaggttcaagacgg tagctcgcacgatgtagctg	*Homo sapiens cytochrome c-1*	NM_001916.3	Generation of precursor metabolites and energy (metabolism)
EIF4A2 (246 bp)	ctccgcggattataacagagaaga gtggctgtcttgccagtacct	*Homo sapiens eukaryotic translation factor 4A2*	NM_001967.3	Transcriptional/translation regulation (regulation)
GAPDH (222 bp)	tcgccagccgagccacatc cgttctcagccttgacggtgc	*Homo sapiens glyceraldehyde-3-phosphate dehydrogenase*	NM_002046.3	Carbohydrate metabolism (metabolism)
GNB2L (224 bp)	gagtgtggccttctcctctg gcttgcagttagccaggttc	*Homo sapiens guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1*	NM_006098.4	Protein kinase, receptor (signal transduction)
HPRT (226 bp)	cagccctggcgtcgtgattagt ccagcaggtcagcaaagaat	*Homo sapiens hypoxanthine phosphoribosyltransferase 1*	NM_000194.2	Purine nucleotide generation (metabolism)
MUC1 (181 bp)	catgcaagctctaccccagg taccttctcggaaggccaga	*Homo sapiens mucin1, cell surface associated*	NM_001018017.2	Protection of epithelial surfaces (cytoskeletal)
RPL13A (126 bp)	cctggaggagaagaggaaagaga ttgaggacctctgtgtatttgtcaa	*Homo sapiens ribosomal protein L13a*	NM_012423.3	Ribosomal protein (transcription)
TUBB (177 bp)	cttcggccagatcttcagac agagagtgggtcagctggaa	*Homo sapiens tubulin, beta 2A class IIa*	NM_001069.2	Mitosis, intracellular transport, neuron morphology, motility
VIM (831 bp)	aacttaggggcgctcttgtc tcgttggttagctggtccac	*Homo sapiens vimentin*	NC_018921.2	Intermediate filaments, mesenchymal specific (cytoskeletal)
YWHAZ (222 bp)	ttggctgaggttgccgctgg gttcagcaatggcttcatcaaaa	*Homo sapiens tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide*	NM_003406.3	Protein kinase C signalling pathway (signal transduction)

with direct comparison of their transcription levels (Fig. 1). The gene expression measured in FFPE tissue always showed higher C_t values than fresh tissue or cell samples, reflecting a lower mRNA expression level. Moreover, in the tissue and cell sample groups, several genes showed variable expression in comparison with the FFPE tissue group. The three highest expressed genes were, for fresh frozen tissue (meningioma and dural tissue): *ACTB* (C_t = 15.48 ± 2.01), *RPL13A* (C_t = 15.58 ± 1.94) and *B2M* (C_t = 16.77 ± 2.16); for cell culture samples *B2M* (C_t = 18.47 ± 4.86), *ACTB* (C_t = 20.71 ± 5.06), *YWHAZ* (C_t = 21.63 ± 2.83), for FFPE tissue *B2M* (C_t = 24.78 ± 1.07), *MUC1* (C_t = 25.09 ± 0.93) and *RPL13A* (C_t = 25.61 ± 2.90). The lowest expression showed *HPRT* in fresh frozen tissue (C_t = 22.04 ± 1.56) and cell culture samples (C_t = 25.75 ± 3.01) and in FFPE tissues *EIF4A2* (C_t = 30.05 ± 1.02). Between tumour and non-pathological tissue, we found significant differences in the tissue groups (fresh and FFPE tissue) but not in the cell culture samples (Fig. 1).

Expression Stability and Validation of Reference Genes

To analyse the ranking of the housekeeping genes, the geNorm and NormFinder algorithms as well as the ΔC_t method and Pearson correlation analysis were used. Each of these analysis algorithms assesses the stability of genes from a specific perspective.

Expression stability calculated by the NormFinder algorithm considers a combination of the inter- and intra-group variance for the tested genes. NormFinder showed differences in stability of gene expression across all tested groups as well as between tumour and non-pathological tissue. The expression stability was determined by its stability value (*S*), coding for high stability at a value near to "0". Analysis of tissue originating from dura and meningioma demonstrated that the most stably expressed genes, with low *S* values were for, fresh tissue samples *TUBB* (*S* = 0.457), *HPRT* (*S* = 0.545) and *B2M* (*S* = 0.564); for cell culture samples *ATP5B* (*S* = 0.087), *TUBB* (*S* = 0.147) and *CYC1* (*S* = 0.356); and for FFPE tissue

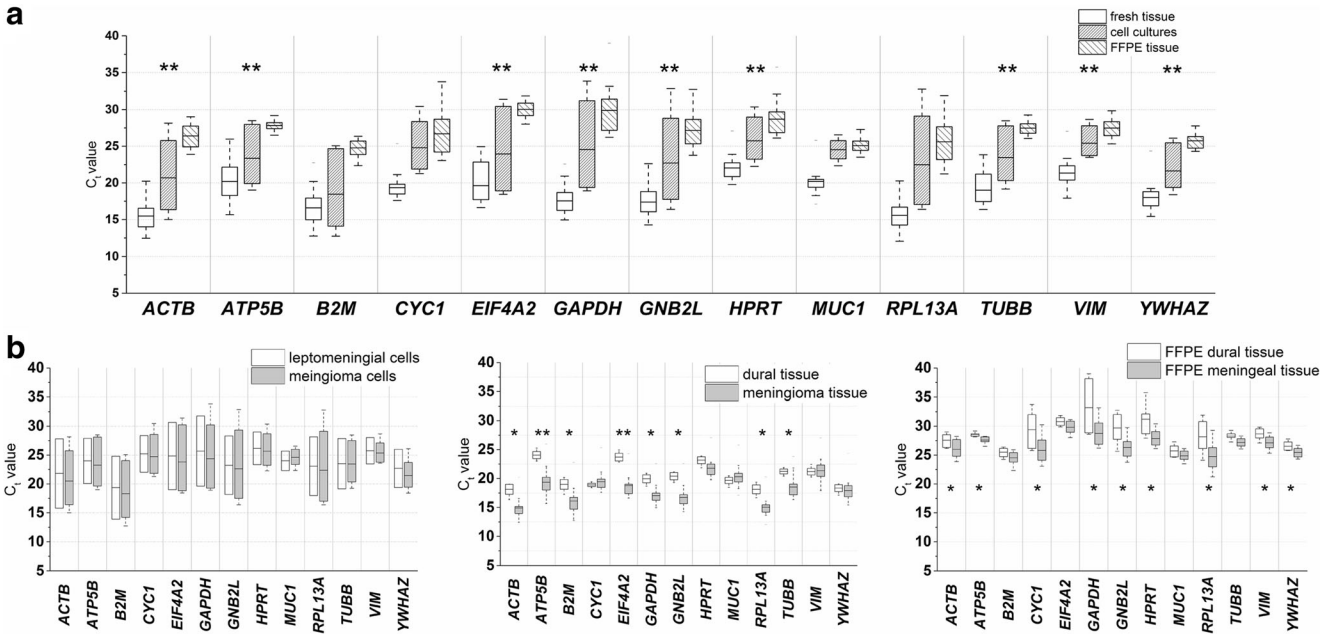

Fig. 1 Expression levels (C_t values) of all pre-selected genes for internal normalisation. The graphs show lowly, moderately and highly expressed genes with the variation of all C_t values for the different experimental settings (**a**) and analysed tissue specimens (meningioma and non-pathological meningeal tissue) (**b**)

samples *ACTB* (S = 0.445), *VIM* (S = 0.547) and *B2M* (S = 0.880) (Fig. 2). Further analysis involved comparing meningioma and dural tissue subgroups. For each subgroup as well as analysis group, we found differences in determination of the most stable gene (Fig. 2). The most stably expressed combinations of housekeeping genes for the mixed group of meningioma and normal meningeal tissue were *HPRT/RPL13A* (S = 0.172) for fresh tissue, *TUBB/ATP5B* (S = 0.087) for cell culture samples and *GNB2L/B2M* (S = 0.335) for FFPE tissue.

Use of the geNorm algorithm determined the most stable genes from a set of tested candidate reference genes in a given sample panel and enabled the elimination of the worst candidates [22]. Based on the geometric mean, a gene expression normalisation factor (M) can be calculated for each sample, resulting in the most stable pair of reference genes with the lowest M value. The candidate genes with the highest stability were, for fresh tissue *ACTB* and *GAPDH* (M = 0.559), for cell culture samples *TUBB* and *ATP5B* (M = 0.742) and for FFPE tissue *TUBB* and *YWHAZ* (M = 0.336) (Fig. 2). As also seen in NormFinder analysis, the best candidates for normalisation varied between the meningioma and non-pathological subgroups (Fig. 2). For example, in the fresh tissue analysis group, *VIM* was the most stable gene for dural tissue (M = 0.083) but one of the least stable for meningioma tissue (M = 1.380).

To confirm both sets of results, Pearson correlation analysis was additionally performed. Here the expression of the genes at non-normalised levels was analysed. The expression levels were determined by log-squared transformation of the relative expression value R and plotted against the mean C_t value of the complete set of transcripts. Gene stability was determined by linear regression analysis. The coefficient of determination (r^2) was calculated to validate the ability of every single transcript to estimate the expression value of the others. The coefficient of determination reflects how much of the data representing expression accords with the expression of the remaining candidate reference genes under specific conditions. For example the coefficient of determination for *YWHAZ* showed a value of 0.658, meaning that 65.8% of the data representing *YWHAZ* expression accorded with the expression of the remaining 12 candidate reference genes under cell culture conditions. Figure 3a shows the inverted coefficient of determination of this value; near − 0 defines stable genes and values > 0.5 indicate less stable gene expression. Most genes showed values < 0.5; therefore, the majority of the candidate reference genes were defined as stable according to Pearson correlation analysis. Only *VIM* and *MUC1* in the fresh tissue group were defined as less stable. The values for the coefficient of determination (r^2) ranged from 0.388 to 0.903 for fresh tissue (mixed dural and meningioma tissue), from 0.658 to 0.984 for cell culture samples, and from 0.601 to 0.970 for FFPE tissue. The highest correlation we found for *HPRT* (r = 0.950, p = 0.000), *ACTB* (r = 0.936, p = 0.000) and *TUBB* (r = 0.927, p = 0.000) in fresh tissues; for *GAPDH* (r = 0.989, p = 0.000), *TUBB* (r = 0.992, p = 0.000) and *ATP5B* (r = 0.985, p = 0.000) in cell culture samples, and

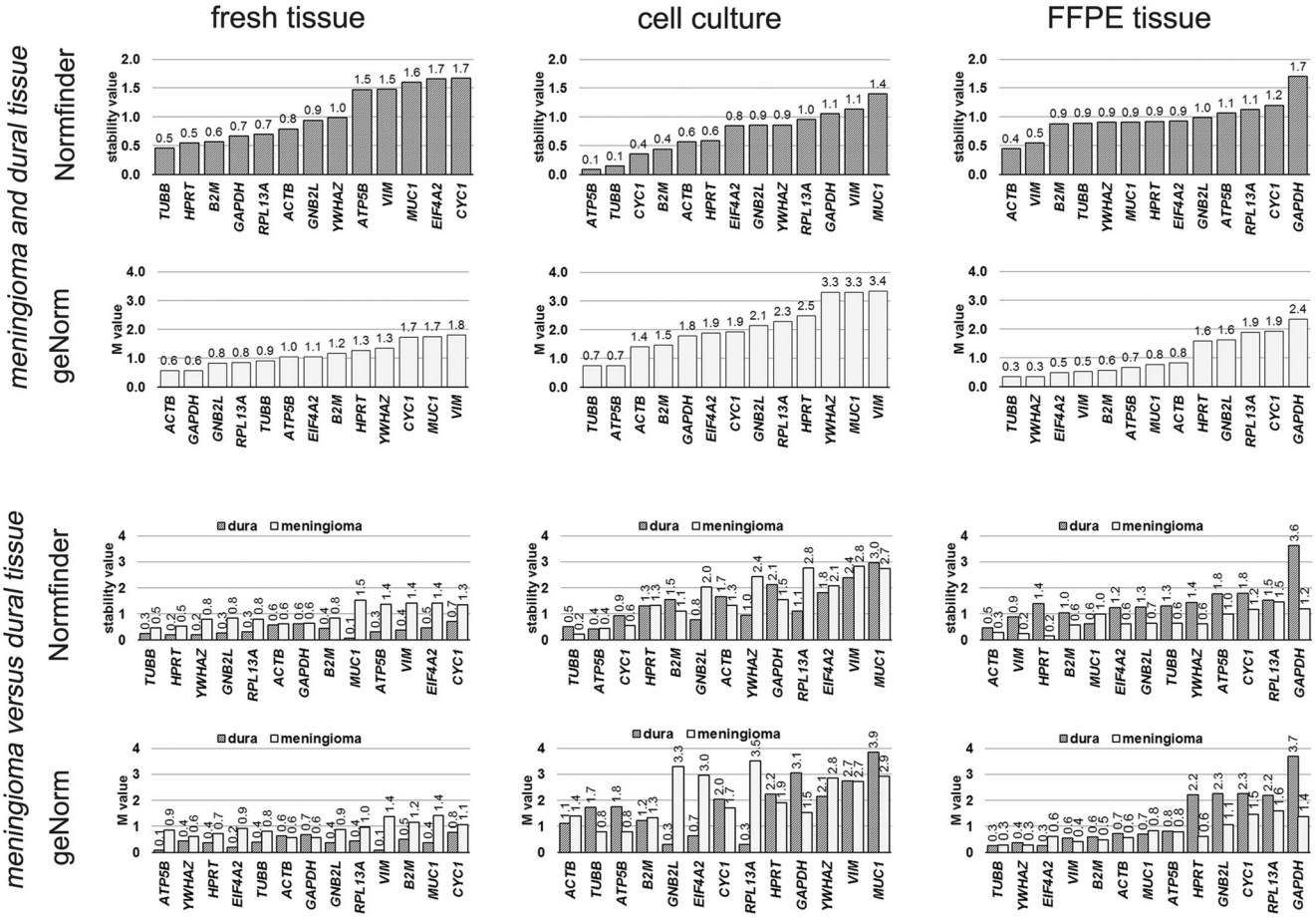

Fig. 2 NormFinder and geNorm analyses. The graphs show stability values (*S*) evaluated by Normfinder algorithm and *M* value as results of the geNorm analysis for all analysed tissue/cell groups together (above) as well as for analysis of pathological and control group (below)

CYC1 ($r = 0.985$, $p = 0.000$), *HPRT* ($r = 0.971$, $p = 0.000$) and *GNB2L* ($r = 0.970$, $p = 0.000$) in FFPE tissue (Fig. 3b). These were the best candidate reference genes in comparison with the other tested candidate genes.

The fourth method used to evaluate for the most appropriate reference genes was a simple ΔC_t method [23]. This method compared relative expression of pairs of genes within each sample. A comparison of ΔC_t value determined in various samples (standard derivation) indicated whether a gene was stably expressed or coregulated, taking all candidate reference genes into consideration and comparing all possible combinations of genes within each sample group. After estimation of the average standard derivation (SD_m), the following genes were demonstrated to demonstrate the least variability: *ACTB* ($SD_m = 1.12$), *HPRT* ($SD_m = 1.13$) and *GAPDH* ($SD_m = 1.14$) in fresh tissue; *TUBB* ($SD_m = 1.89$), *ATP5B* ($SD_m = 1.93$) and *B2M* ($SD_m = 2.15$) in cell culture samples; and *VIM* ($SD_m = 1.15$), *ACTB* ($SD_m = 1.19$) and *TUBB* ($SD_m = 1.26$) in FFPE tissue (Fig. 4a). After separation into tumour and non-tumour tissue, we found lower mean standard derivations and other genes were defined as the most stable (Fig. 4b).

Selection of the most Stable Gene(s) for Normalisation and Ranking

As shown, raw data were analysed by four different statistical models: on the one hand, by the two algorithms NormFinder [3] and geNorm [22], by the ΔC_t method [23] that ranked the candidate genes according to their expression stability, and lastly by Pearson correlation analysis utilising the individual coefficients of determination.

Each of these analytical methods is based on particular statistical calculations. NormFinder calculated the stability value for each pre-selected gene by comparison of inter- and intragroup variations, being highly sensitive for stability according to internal normalisation. Low transcript stabilities with an *S* value of > 1, representing a high variance and low expression steadiness, were detected for *GAPDH*, *RPL13A*, *ATP5B*, *VIM*, *MUC1*, *EIF4A2* and *CYC1* in one or two of the tested analysis groups, but not in all. High transcript stabilities with low *S* values of < 1 were found for *TUBB*, *HRPT*, *ACTB*, *GNB2L* and *YWHAZ* in all analysis groups. In conclusion, the most stable expressed reference genes for all groups were *TUBB*, *ACTB* and *B2M*. To avoid false positive results, we further determined expression stability by a

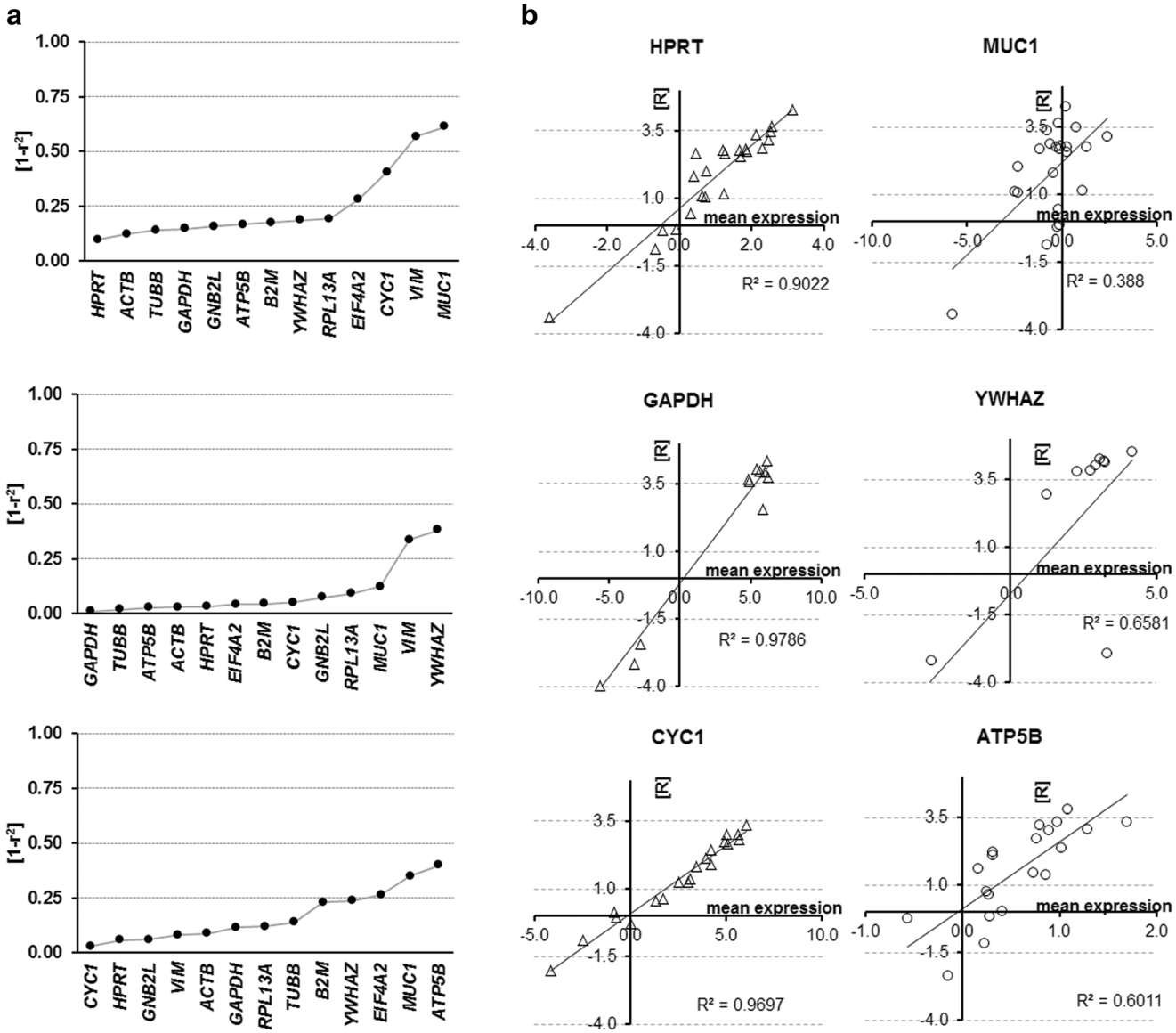

Fig. 3 Pearson correlation analysis. **a** Inverted coefficient of determination for fresh tissue (above), cell culture samples (middle) and FFPE tissue (below). **b** The genes with the highest correlation to mean expression of all tested genes as well as the genes with the least correlation of equal sample group

pairwise correlation analysis with the geNorm algorithm, calculating gene stability by determination of the normalisation factor *M*. These calculations were performed under the precondition of non-coregulation of all selected candidate genes. Finally, grading all 13 tested genes according to their normalisation factors revealed values of $M > 0.3$ in all cases. $M < 0.3$ indicates high expression stability under different conditions. We found varying *M* value ranges across the analysis groups. We detected $0.6 < M > 1.8$ for fresh tissue samples, $0.7 < M > 3.4$ for cell culture samples and $0.3 < M > 2.4$ for FFPE tissues. The most stable candidate reference genes determined by geNorm were *TUBB*, *ACTB* and/or *ATP5B* across all experimental groups.

Analysing pairwise variations based on the ΔC_t method is commonly used for evaluation of gene expression performed by qPCR. Here, the determined mean standard derivations across each gene pair and sample should show optimally be low, demonstrating high stability. Across all analysis groups, *TUBB* and *ATP5B* were among the top five most stable candidate genes.

Subsequently, Pearson correlation revealed *HPRT*, *TUBB* and *CYC1* to be the genes with the most positive correlation against all pre-selected candidate genes in the relevant groups.

Additional ranking analysis of all tested genes and using each calculation algorithm, allowed an individual comparative rank to be assigned. For identification of the most stable genes, these rank numbers were determined by the geometric mean as mean ranking values (r_m). This ranking revealed *HPRT* ($r_m = 2.45$), *TUBB* ($r_m = 2.78$) and *GAPDH* ($r_m = 3.13$) as the most stably expressed reference genes in the combined group of all

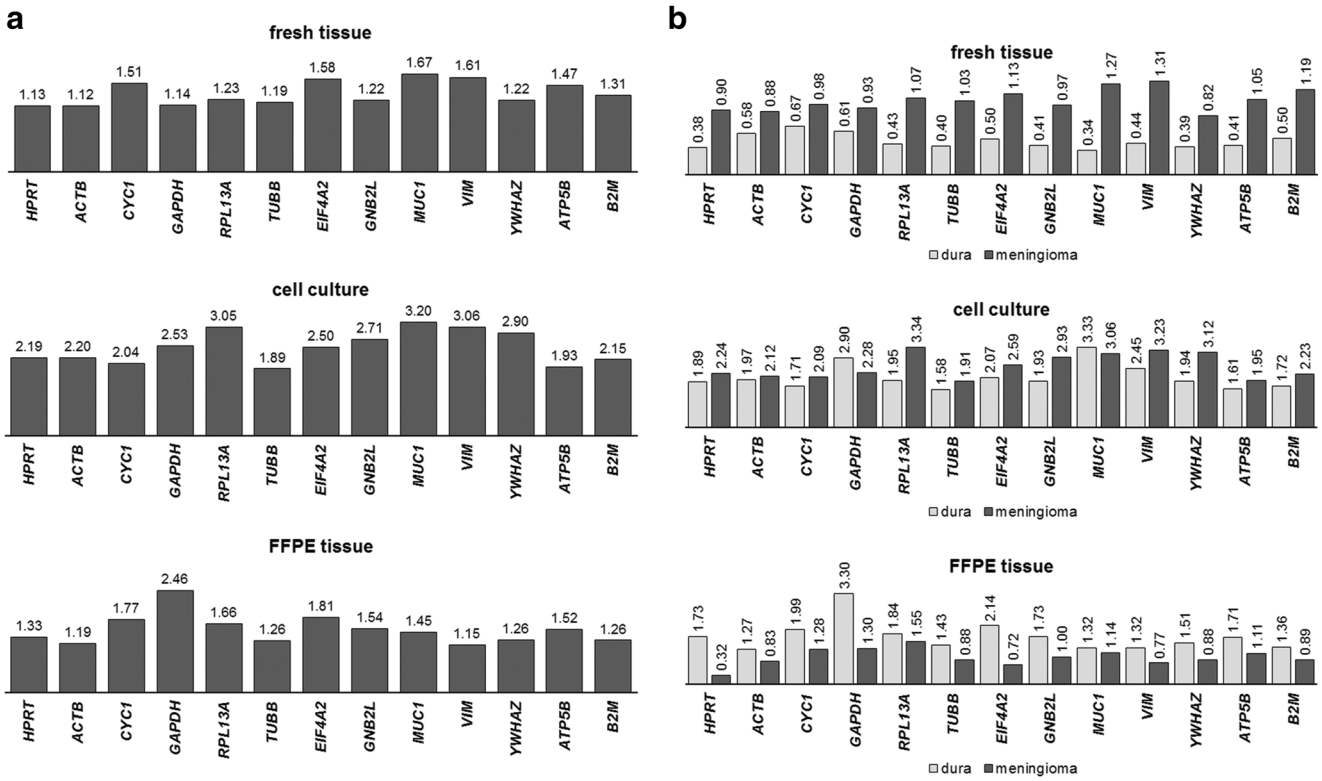

Fig. 4 Results of ΔC_t method. The graphs show mean standard derivation of mean ΔC_t for all analysed tissue/cell groups together (**a**) as well as for analysis of pathological and control group (**b**)

meningioma and meningeal fresh tissue samples. For mixed cell culture samples, we evaluated *TUBB* (r_m = 1.41), *ATP5B* (r_m = 2.00) and *ACTB* (r_m = 4.05) to be the most stable candidate genes by ranking analysis. For the FFPE tissue samples, we found *VIM* (r_m = 2.632) to be the most stable gene followed by *ACTB* (r_m = 2.99) and *TUBB* (r_m = 4.28). Across all experimental groups, *TUBB* was consistently among the three most stable expressed candidate genes (Fig. 5).

Discussion

Quantitative polymerase chain reaction (qPCR) is an effective method to measure gene expression, especially when analysing variations between different histological tissue samples and experimental groups [24]. In this study, samples from three different experimental settings were analysed by qPCR to find a suitable reference gene for normalisation. We tested 23 fresh tissue samples of meningioma tissue encompassing all WHO grades and dural tissue, 12 samples of primary meningioma and dura/arachnoidea cell cultures and 20 samples of FFPE meningioma and dural tissue with potentially different individual gene profiles.

To avoid misinterpretation of qPCR findings, it is essential to normalise the results of the tested target genes [27, 28]. For this normalisation, RNA values of the target genes should be corrected for sample-to-sample variations by amplifying them along an internal reference [29]. A crucial precondition for an optimal internal standard gene is its nearly constant expression in the analysed tissues of the same organism at all stages of development, unaffected by different experimental settings or treatments. In conclusion, the ideal reference gene should be constantly expressed in all situations [28, 30]. Among others, the most commonly used reference genes are therefore *GAPDH* and the ribosomal RNAs [27]. However, to date and to the authors' knowledge, there have been no relevant reports on unregulated candidate reference genes in human meningiomas that can reliably be used to provide an internal standard for other experimental settings. Therefore, it was the aim of this study to identify the most stably expressed genes from a list of 13 candidate reference genes, serving as internal controls in future meningioma research.

Within the sphere of current brain tumour research, relatively little is known about gene expression in untreated meningioma tissue, FFPE tissue or meningioma cell cultures. There are several studies on the molecular characterisation of human meningiomas performed using genomic array analysis, but quantitative PCR has seldom been utilised [21]. To bridge this gap, this study tested a set of candidate reference genes, which are common in a number of tissues, such as *ACTB*, *GAPDH*, *RPL13A*, *HPRT*, *B2M* or *TUBB*, complemented by genes for normalisation, which were more specific to meningiomas or reflect cancerous transformation, like *VIM* and *MUC1* [31, 32].

For detection of the most stable expression of the candidate reference genes, necessary for further normalisation, the two software algorithms (NormFinder and geNorm),

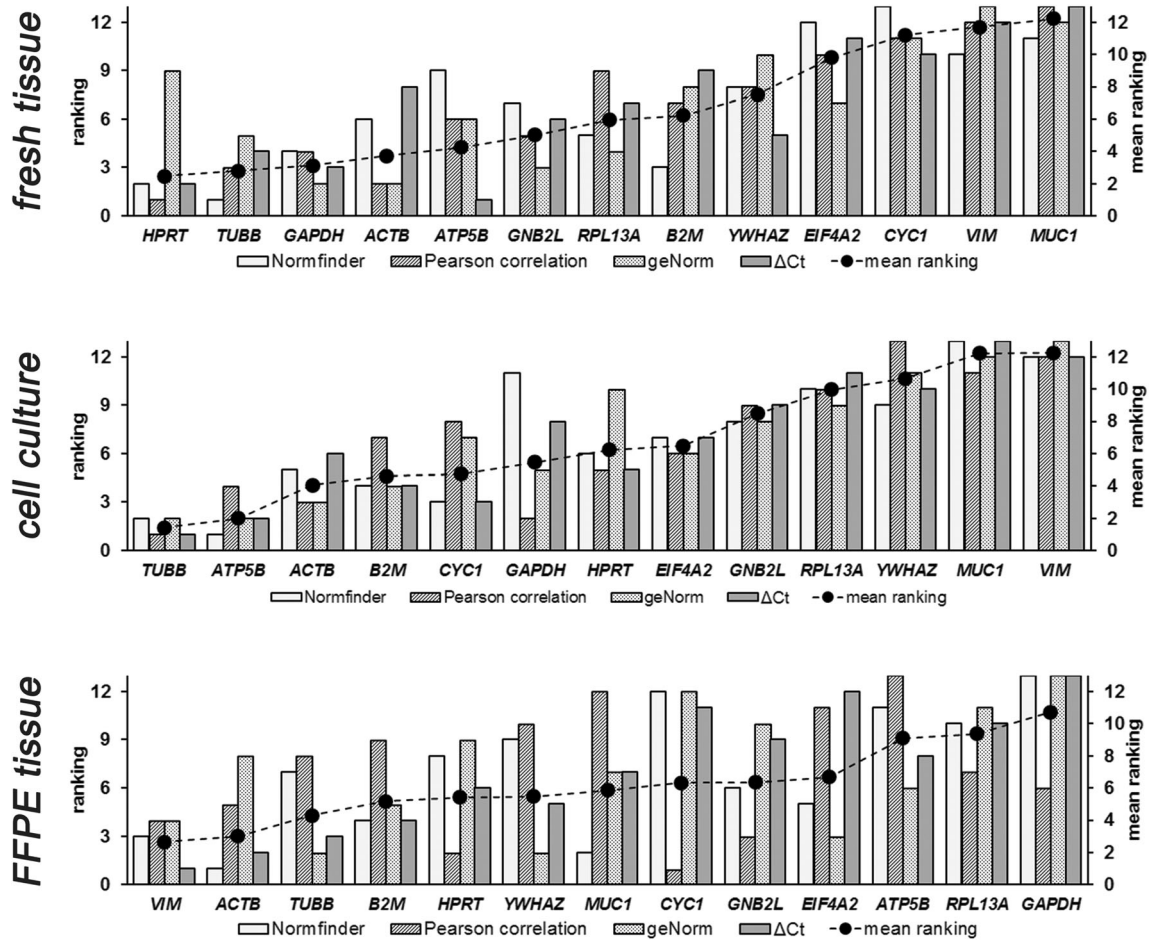

Fig. 5 Ranking analysis. The graphs show the result of the final ranking analysis of all four processed evaluation methods for all sample groups. The point reflects the mean ranking of the candidate reference gene

complemented by Pearson correlation, the ΔC_t method and a final ranking analysis, were applied.

In conclusion, all different data assessments and analysis algorithms provided complementary results, with *TUBB*, *ACTB* and *HPRT* as the most stably expressed genes across all sample groups of mixed meningioma and dural tissue. Still, there were differences between the experimental settings. The "viable" tissue or cell samples generally revealed commonly utilised housekeeping genes to be the most stable. For the tested fresh tissue samples, *HPRT*, *TUBB*, *GAPDH* and *ACTB* followed by *ATP5B* were the most stable, with *YWHAZ*, *EIF4A2*, *CYC1*, *VIM* and *MUC1* the least stable candidate genes. However, the different analysis methods showed large variations in the ranking of the most stably expressed gene. In light of these findings, it is important to note that tissue samples were frozen directly after neurosurgical resection. This introduces a caveat in that processed samples showed high heterogeneity in cell composition and contamination with other tissue structures. In cell culture samples, the genes *TUBB*, *ATP5B*, *ACTB*, *B2M* and *CYC1* were demonstrated to be housekeeping genes suitable for normalisation. The variations seen when the varying measurement algorithms utilised were relatively homogeneous. One explanation for this effect may be the cultivation-dependent isolation of only a few cell types (mature meningioma cell, progenitor cells, senescent cells) without no residual blood or vessel cells. In the literature, little is known about reference genes in cell culture systems and nothing is known about this in the context of primary human meningioma cells. Still, the importance of selection of the accurate gene for normalisation has been demonstrated in colon cancer cells and in tissue [33] and also in ovarian cancer cell lines [34]. The third analysis group, FFPE meningioma and meningeal tissue, showed *VIM*, a cancer-specific marker, to be most stably expressed, followed by *ACTB*, *TUBB*, *B2M* and *HPRT*, whereas *GAPDH* showed the worst value for stability.

While we were able to isolate intact mRNA from FFPE tissue, it is known that this process is often unsuccessful owing to the chemical modification (for example, oxidative deamination) and RNA degradation that takes place during fixation. Despite such degradation, however, small fragments that are suitable for amplification may remain intact in FFPE samples [35, 36]. Furthermore, the ability to analyse mRNA from FFPE tissue is beneficial, particularly for retrospective studies, owing to the plentiful nature of archival FFPE samples [37].

This study shows that there are some individual differences in gene expression stability depending on the statistical testing method and the particular experimental setting used. This highlights the need for optimal a priori study design, incorporating specific testing algorithms and appropriate tumour tissue and control selection.

One relevant limitation of this study is the pre-selection of candidate reference genes for evaluation, a step that potentially excluded other, better reference genes. A further caveat is the small sample size.

The strength of this study is the analysis of both meningioma and meningeal tissue which has not been done before to our knowledge. Another important aspect is our analysis across three different experimental settings (fresh tissue, cell culture samples and FFPE tissue), which opens up the possibility for a large variety of future studies including retrospective analyses, in vitro experiments and transcription analysis from a defined patient cohort. The utilisation of four different analytical methods enhances the strength of the results and our determination of the most stable reference genes on the basis that the establishment of reference genes for normalisation of target genes should optimally incorporate at least three stability analysis methods [34].

Conclusion

Validation of unregulated and stably expressed reference genes for normalisation of quantitative PCR data in meningioma and non-pathological meningeal tissue is essential for studies of target gene expression in meningiomas. In this study, we analysed a set of 13 pre-selected candidate reference genes for housekeeping in surgical tissue specimens, in cell culture specimens and in FFPE tissue samples of meningioma and non-pathological dural and arachnoid tissue together.

Statistical evaluation of the expression analysis was performed using NormFinder and geNorm algorithms, supplemented by Pearson correlation and ΔC_t analysis. Although the individual testing results differed to some degree, the combination of all four evaluation methods and a final ranking analysis delivered highly robust results.

In conclusion, the highest expression stability and therefore the best suitability as meningioma reference genes for internal standardisation, was *HPRT* for fresh tissue, *TUBB* for cell culture samples and *VIM* for FFPE tissue.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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