Identification of Valid Reference Genes for Reliable RT-qPCR in Human Normal and Cancer Brain Cell Lines

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ABSTRACT: Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) has emerged as robust assay that provides absolute and relative quantification of the MRNA transcription level. The expression of interest gene is usually normalized to an internal standard known as the reference gene. Reference genes are genes that are expressed constantly in all cells to maintain essential cellular functions. However, expression of these genes may vary in distinct tissue types and under certain conditions. Thus, it is imperative to identify the most stably expressed reference genes in each experimental design for reliable RT-qPCR. In this study, we evaluated six candidate reference genes, glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyl transferase 1 (*HPRT1*), 60s ribosomal protein large P1 (*RPLP1*), TATA box binding protein (*TBP*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*) and eukaryotic initiation factor 4A (*elF4A*). To determine the expression levels of these genes, the RT-qPCR was performed in human brain cell lines: normal glial cell line (SVG p12) and cancer glioma cell line (DBTRG-05MG). The qPCR data was analyzed using NormFinder and Bestkeeper. The analyses revealed the most stably expressed gene was *TBP* while *RPLP1* was ranked as the least stable expressed gene. Therefore, we proposed to select *TBP* and *RPLP1* as the valid reference gene for comparison between normal and cancer brain cell lines.

Keywords: Glioma, Reference genes, RT-qPCR

Introduction

Gene expression analysis provides insight of internal blueprint on genetic information which leads to the identification of a specific gene, which is being turned on to display a particular phenotype or pathological state of the organism. Most mechanisms regulate the gene expression via messenger RNA (mRNA) transcription. The mRNA can be quantified using reverse transcription (RT)-quantitative Polymerase Chain Reaction (qPCR). RT-qPCR is the most well-known technique for absolute and relative quantification of the MRNA transcription level. This method offers a broad range of advantages such as greater sensitivity, higher specificity, cost-effective and high-throughput as compared to semi quantitative PCR and Northern blot.

The expression of interest gene is usually normalized to an internal standard known as the reference gene. Reference genes are genes that are expressed constantly in all cells to maintain essential cellular functions (Watson *et al.*, 1965). However, expression of these genes may vary in distinct tissue types and under certain conditions (Wong and Medrano, 2005). In 2000, Warrington and co-workers described the expression profile of a total of 7,128 genes in 11 distinct human normal adults and fetal tissues using GeneChip probe arrays. Of these, 535 reference genes were expressed in each of 11 human adult and fetal tissues. A later study by Hsiao *et al.* (2001) analyzed the expression of 7,070 unique genes using microarray. A total of 451 reference genes were turned on in all 19 different tissue types of 59 human samples, in which 358 of the reference genes were identical as reported previously (Warrington *et al.*, 2000). These studies showed that different types of tissue exhibit specific expression profile of reference genes. Thus, there is no universal reference gene which could be used in all studies without prior verification. Since the validity and accuracy of RT-qPCR are highly dependent on the selection of reference gene, therefore it is imperative to identify the ideal candidate reference genes prior to RT-qPCR. However, despite its importance, majority of the gene expression studies did not validate the reference gene prior to RT-qPCR. Generally, these studies applied either one of the most classical reference genes such as glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) and beta actin (*ACTB*) without a thorough selection of the most stable internal controls.

As a consequence, the normalization using non-validated reference genes may introduce errors (Gutierrez *et al.*, 2008). To solve this problem, here, we evaluated six frequently used candidate reference genes with the aim to identify the most appropriate reference genes for RT-qPCR analysis of human brain glial and glioma cell lines.

The six candidate reference genes are glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyl transferase 1 (*HPRT1*), 60s ribosomal protein large P1 (*RPLP1*), TATA box binding protein (*TBP*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*) and eukaryotic initiation factor 4A (*elF4A*).

Glioma, which arises from abnormal glial cells, including astrocytomas, oligodendrogliomas, oligoastrocytoma and ependymomas is the most common malignant brain tumor in adults. Glial cells play a variety of essential roles in the brain, such as "supporting cells" for neurons, nutrient and oxygen transportation, as well as neurotransmitters shuttle in and out of a neuron. Glioma may results from sequential accumulation of several genetic instabilities at the chromosomal level or at the gene expression level (Sulman *et al.*, 2009). Thus, identification of the differently expressed genes in brain cancer cells but not in normal brain cells may provide better understanding on the fundamental molecular basis leading to malignant transformation and glioma progression. In this direction, several studies have been focused on identifying the differentially expressed genes in glioma using RT-qPCR (Haque *et al.*, 2007; Maier-Hauff *et al.*, 2007; Scrideli *et al.*, 2008).

Materials and Methods

Human glial cell samples (SVG p12) and human glioma cell samples (DBTRG-05MG) were purchased from ATCC, USA. The SVG p12 cells were cultured in ATCC-formulated Eagle's Minimum Essential Medium (GIBCO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) Penicillin-Streptomycin (GIBCO, USA). The DBTRG-05MG cells were cultured in complete growth medium RPMI 1640 (GIBCO, USA) supplemented with 10% FBS and 1% Penicillin-Streptomycin. All cells were cultivated in incubator under 5% CO_2, at 37^0C until the cells confluent.

Prior to RNA extraction, cells were harvested and centrifuged at 1,500 rpm for 5 minutes at room temperature. RNA was extracted from a total of 10^7 cells using the RNeasy Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The concentration and $A_{260}/_{280}$ ratio of RNA were determined using Eppendorf BioPhotometer plus spectrophotometer (Eppendorf, Germany). A ratio of $A_{260/280}$ approximately 2.0 or greater is generally accepted as pure RNA. The integrity of total RNA was determined by 1% agarose gel electrophoresis.

For cDNA synthesis, 5 µg of total RNA were reverse transcribed using ReverseAid Minus First Strand cDNA Synthesis kit according to the manufacturer's protocol (Thermo Scientific, USA). The reaction was incubated for 5 minutes at 25^0C followed by 60 minutes at 42^0C and 70^0C for 5 minutes.

Primers were designed using Primer3 software and blasted against NCBI databases (**Table 1**). Each primer pair flanking the exon-exon boundary were designed to avoid genomic DNA amplification that possibly present in the sample.

Table 1: Primer pair sequences for each reference gene used in the study

Gene	Forward primer [5'-3'] Reverse primer [5'-3']
Glyceraldehyde-3-phosphate (*GAPDH*)	CCA AGG TCA TCC ATG ACA AC
	ACA GTC TTC TGG GTG GCA GT
Hypoxanthine guanine phosphoribosyl transferase (*HPRT*)	TGA GGA TTT GGA AAG GGT GT
	CCT CCC ATC TCC TTC ATC AC
60s ribosomal protein large P1 (*RPLP1*)	AGC CGG TGT AAA TGT TGA GC
	CAG ATG AGG CTC CCA ATG TT

TATA box binding protein (*TBP*)		TTC GGA GAG TTC TGG GAT TG
		GGA TTA TAT TCG GCG TTT CG
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*)		CCG TTA CTT GGC TGA GGT TG
		TTG CAT TTC CTT TTT GCT GA
Eukaryotic initiation factor 4A (*elF4A*)		GAA GCT GGA TTA CGG ACA GC
		GCA CGT GTA CTT AGG CTT CT

The RT-qPCR amplification was carried out in an optical 96-well plate with an Applied Biosystems 7500 Real-Time PCR system, using SYBR Green to monitor double stranded DNA synthesis. Briefly, the reactions contained 1X Power SYBR Green Master Mix (Applied Biosystems, USA), 10 ng of cDNA template and 10 nM of each primer pair in a total reaction volume of 25 µL. Amplification was performed by an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation step at 95°C for 15 seconds and both annealing step at 60°C for 1 minute and extension step at 72°C for 30 s. For each experiment, a non-template reaction was used as a negative control. Amplicon dissociation curves (melting curves) were recorded after cycle 40 by gradually heating from 60°C to 95°C. PCR efficiency (E) was calculated based on the data obtained from the exponential phase of each individual amplification plot via equation $E = [10^{(-1/slope)}-1] \times 100$ (Ramakers *et al.*, 2003).

To compare the transcription level of the reference genes, the average cycle threshold (Ct) value of each duplicate reaction was used as input for subsequent analysis using the BestKeeper and NormFinder programs. The BestKeeper computed the gene expression variation for the six reference genes in all the samples based on Ct. It sets up a pairwise correlation coefficient (r) between each gene and the BestKeeper index is the geometric mean of the Ct values of all candidate reference genes when grouped together. Sample integrity value was calculated to remove data which obscure the accuracy of the reference gene evaluation. Standard deviation (SD) of the Ct values between the entire data set was

calculated. The most stably expressed reference gene exhibited the highest correlation coefficient with BestKeeper index. The NormFinder estimated the overall expression variation of the candidate reference genes and the variation between sample subgroups. A stability value for each gene represents variation in expression within samples and between groups. The lower stability value indicated lower intragroup and intergroup variations and higher stability. It also provided the best combination of two reference genes for each experimental set, which have a corresponding lower stability value.

Results

Total RNA was isolated from the human glial cell line, SVG p12 and glioma cell line DBTRG-05MG. The purity of RNA from SVG p12 and DBTRG-05MG, which was estimated from $A_{260/280}$ ratio, was 1.72 ± 0.06 and 1.76 ± 0.04 respectively. The $A_{260/280}$ ratio was lower than the acceptable range indicated the contamination of DNA or protein, which absorb strongly at 280 NM. Therefore, integrity of total RNA was further verified on 1% agarose gel. The ribosomal RNAs, 28S rRNA and 18S rRNA appeared as sharp bands without smear (**Figure 1**). The 28S rRNA band was approximately twice as intense as the 18S rRNA band indicated that all the RNAs were intact and of good quality for RT-qPCR analysis.

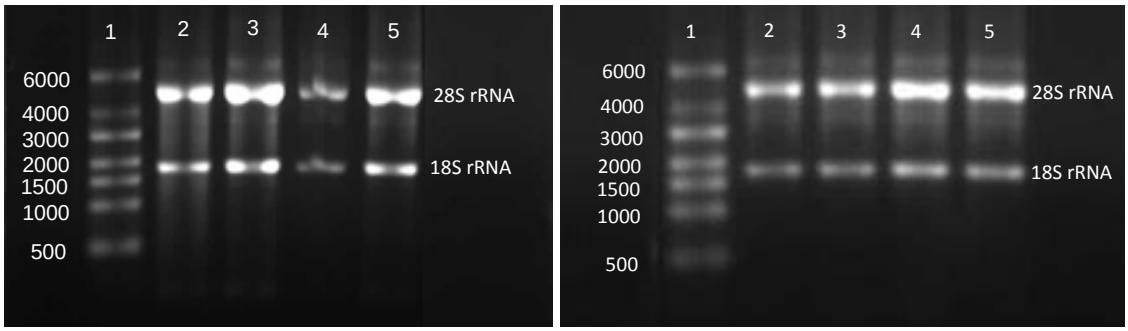

Figure 1: Non-denaturing agarose gel electrophoresis of total RNA isolated from SVG p12 (left) and DBTRG-05MG (right). The 28S and 18S rRNA bands were indicated. Lane 1: RNA ladder; lanes 2, 3, 4 and 5: intact RNA samples

The amplification efficiency of each primer pair was successfully determined using serial dilution curves. The amplification efficiency of all the samples ranged from 93.43 to 114.24% with a positive correlation coefficient (R^2) ranged from 0.994 to 0.999 (**Table 2).**

The amplification efficiency test showed that all primer pairs consistently performed throughout the amplification.

Table 2: The amplification efficiency (E) and correlation coefficient (R^2) of reference genes used in the study.

Gene	Efficiency, $E=[10^{(-1/slope)}-1] \times 100$	Correlation coefficient (R^2)
GAPDH	112.38 %	0.995
HPRT1	99.66%	0.999
TBP	114.24%	0.999
RPLP1	93.43%	0.996
eIF4A	99.66%	0.994
YWHAZ	107.62%	0.990

The expression levels of six candidate reference genes were relatively similar across SVG p12 and DBTRG-05MG. The expression level of candidate gene was inversely proportional to the number of cycle threshold. *GAPDH* had the highest level of expression with low cycle numbers within the range $18 < Ct < 20$ cycles. While the gene with the lowest expression in different cell lines is *TBP* with cycle numbers > 25 cycles. Moreover, the other candidate genes had similar cycle numbers and hence all demonstrated moderately low expression in both SVG p12 and BDTRG-05MG cell lines (**Figure 2**).

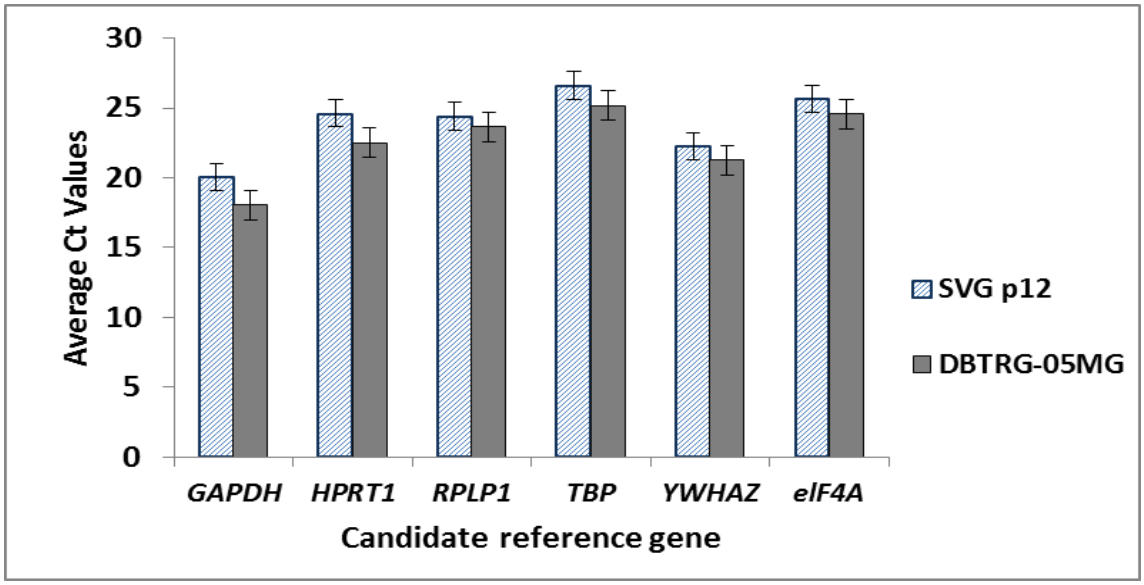

Figure 2: Expression levels (cycle threshold, Ct value) of candidate reference genes in human normal glial cell line, SVG p12 and glioma cancer cell line, DBTRG-05MG. The average Ct values were relatively not much different across both cell lines.

In order to identify the most stably expressed reference genes, the expression data was further analyzed using BestKeeper and NormFinder. NormFinder ranked the set of candidate reference genes based on the overall expression variation by evaluating the intergroup expression variation. From the data, the systematic errors associated with the gene selected for normalization were determined according to the stability value (**Table 3**). TBP showed the lowest stability value which indicated the least intergroup variation with and hence was selected as the most stably expressed genes. NormFinder ranked the *RPLP1* as the least stable reference gene. The analysis also suggested *TBP* and *eIF4A* as the best combined reference genes for normalization between SVG p12 and DBTRG-05MG (**Table 3**).

Table 3: NormFinder analysis for best candidate reference genes between human brain glial and glioma cells. *TBP* exhibited the lowest stability value across the two cell lines. *TBP* and *elF4A* were identified as the best combination of two genes for normalization between SVG p12 and DBTRG-05MG.

SVG p12 vs. DBTRG-05MG		
Ranking	Gene	Stability value
1	*TBP*	0.084
2	*eIF4A*	0.225
3	*YWHAZ*	0.235
4	*HPRT1*	0.337
5	*GAPDH*	0.356
6	*RPLP1*	0.368
Best gene		*TBP*
Best combination of two genes		*TBP* and *eIF4A*
Stability value for best combination of two genes		0.124

GAPDH and *HPRT1* had a standard deviation (SD) more than 1 indicating the gene expression was inconsistent and highly varied (**Table 4**). The other four genes including *TBP*, *RPLP1*, *EIF4A* and *YWHAZ* were considered as consistently expressed gene because of SD less than 1.

Table 4: Descriptive analysis of cycle threshold (Ct) data of candidate reference genes.

	Standard deviation (SD)
GAPDH	1.10
HPRT	1.09
RPLP1	0.63
TBP	0.89
YWHAZ	0.94
eIF4A	0.96

The best correlations were obtained for *TBP* (r= 0.995), *eIF4A* (r= 0.98), *YWHAZ* (r= 0.976), *GAPDH* (r= 0.975), *HPRT1* (r= 0.970) and *RPLP1* (r= 0.954) with a p value of 0.001 (**Table**

5). The descending ranking of most stably expressed genes was as follows: *TBP* **>** *eIF4A* **>** *YWHAZ* **>** *GAPDH* **>** *HPRT1* **>** *RPLP1* (**Figure 3**).

Table 5: Regression analysis of reference genes against BestKeeper index.

	GAPDH	*HPRT1*	*TBP*	*RPLP1*	*YWHAZ*	*eIF4A*
r	0.975	0.970	0.995	0.954	0.976	0.980
p-value	0.001	0.001	0.001	0.001	0.001	0.001

When comparing the stability of candidate reference genes analyzed by both of the software, *TBP* was ranked as the most stably expressed reference gene as compared with other reference genes evaluated in this study (**Table 6**). The top three reference genes resulted from both software also gave a similar ranking sequence with *TBP* followed by *eIF4A* and *YWHAZ*.

Table 6: Ranking of reference genes using NormFinder and BestKeeper programs.

Ranking	Reference gene	NormFinder stability value	Ranking	Reference gene	BestKeeper coefficient correlation (r)
1	*TBP*	0.084	1	*TBP*	0.995
2	*eIF4A*	0.225	2	*eIF4A*	0.980
3	*YWHAZ*	0.235	3	*YWHAZ*	0.976
4	*HPRT1*	0.337	4	*GAPDH*	0.975
5	*GAPDH*	0.356	5	*HPRT1*	0.970
6	*RPLP1*	0.368	6	*RPLP1*	0.954

Discussion

Reference gene is constitutively expressed housekeeping gene that frequently used in RT-qPCR technique for normalization. Ideally, reference genes should exhibit constant expression level across all tissue types and experimental conditions. Unfortunately, recent studies have pointed out that such reference genes do not exist (Warrington *et al.*, 2000;

Hsiao *et al.*, 2001). To avoid dramatically misinterpretations due to the use of unstable internal standard for the RT-qPCR study, the expression level of six reference genes (*GAPDH*, *HPRT1*, *TBP*, *RPLP1*, *YWHAZ* and *eIF4A*) were evaluated between the normal and cancerous cells from the same tissue origin.

Firstly, the quality of RNA was evaluated prior to RT-qPCR in order to reduce the systemic errors of the reaction. This is because RT-qPCR using degraded RNA samples which leads to mRNA cleavage caused inaccurate RNA quantification (Fleige and Pfaffl, 2006). In the present study, the $A_{260}/_{280}$ ratio of RNA isolated from SVG p12 and DBTRG-05MG was lower than the standard range ($A_{260}/_{280}$ ratio $\geq$ 2), which indicated DNA or protein contamination. The RNA integrity was then verified using gel electrophoresis. A sharp band of 28S rRNA and 18S rRNA without smear was clearly observed in each RNA sample, which indicated that all RNAs were intact and of good quality for RT-qPCR study. The gel electrophoresis analysis also ruled out the probability of DNA or protein contamination in the extracted RNA samples. Lower $A_{260}/_{280}$ ratio may due to acidic pH of diluent used during the extraction step which affected the absorbance measurement. Generally, pure RNA has an $A_{260}/_{280}$ ratio ranged between 1.9 and 2.1 in 10 mM Tris-HCl buffer of pH7.5.

Secondly, all the primer pair pairs were designed to span exon-exon junction which ruled out the amplification of residual genomic DNA (gDNA). Competition of residual gDNA in RT-qPCR reduced the amplification efficiency (Freeman *et al.*, 1999). DNase I treatment during the RNA purification also can be used to remove contaminating gDNA.

In this study, NormFinder and BestKeeper were used to select the most stable reference genes. NormFinder ranked the stability (in order of most stable to least stable) as: *TBP*, *eIF4A*, *YWHAZ*, *GAPDH*, *HPRT1* and *RPLP1*. Best Keeper calculated the stability ranking of the six genes with slightly differed order: *TBP*, *eIF4A*, *YWHAZ*, *HPRT1*, *GAPDH* and *RPLP1*. Out of the six reference genes evaluated, *TBP* was ranked as the most suitable reference genes for normalization purposes between human glial and glioma cell lines. NormFinder analysis revealed *TBP* with a lowest stability value which indicated the least intergroup variation. BestKeeper analysis also reported *TBP* with the highest correlation coefficient, which is the most stably expressed across normal and cancerous brain cells. *TBP* gene encoded for general RNA polymerase II transcription factor which specifically binds to

TATA box that present in the regulatory region of several genes. Therefore, *TBP* is expressed in cells and tissues throughout the body. *TBP* has also been reported as the most stably expressed gene in normalization across human normal liver and hepatocellular carcinoma (Fu *et al.*, 2009).

Both NormFinder and BestKeeper suggested that the least stable reference gene for normalization between human glial and glioma cells was *RPLP1*. The *RPLP1* gene encoded for acidic ribosomal phosphoprotein which plays an important role in the elongation step of protein synthesis which may stably expressed in the cancerous cells (Artero-Castro *et al.*, 2011).

NormFinder also calculated the best combination of two reference genes for normalization of human normal and cancer brain cells. In this analysis, *TBP* and *elF4A* were selected as the best combination two genes for normalization of gene expression between SVG p12 and DGTRG-05MG. Normalization based on multiple candidate genes provided the advantage of more accurate normalization calculation than a single gene (Tricarico *et al.*, 2002).

Conclusion

The best candidate reference genes were determined for normalization of gene expression in human normal glial cells and cancerous glioma cells. Our comparative study of several widely used reference genes suggested *TBP* as the ideal reference gene for gene expression studies of human glial and glioma according to both BestKeeper and NormFinder analysis. These results emphasize the significance of utilizing accurate reference genes for glioma gene expression profiling. *RPLP1* was ranked the least stable gene. Thus, *TBP* and *RPLP1* were selected as the best combination of valid reference genes for reliable RT-qPCR analysis between normal and cancer brain cell lines.

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References

Artero-Castro, A., Castellvi, J., García, A., Hernández, J., Ramón y Cajal, S. and Lleonart, M.E. (2011). Expression of the ribosomal proteins Rplp0, Rplp1, and Rplp2 in gynecologic tumors. *Human Pathology,* **42(2)**:194-203.

Fleige, S. and Pfaffl, M.W. (2006). RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine,* **27(2-3)**: 126-139.

Freeman, W.M., Walker, S.J. and Vrana, K.E. (1999). Quantitative RT-PCR: Pitfalls and potential. *Bio Techniques,* **26(1):** 112-122.

Fu, L.Y., Jia, H.L., Dong, Q.Z., Wu, J.C., Zhao, Y., Zhou, H.J., Ren, N., Ye, Q.H. and Qin, L.X. (2009). Suitable reference genes for real-time PCR in human HBV-related hepatocellular carcinoma with different clinical prognoses. *Bio Med Central Research Cancer,* **9**: 49.

Gutierrez, L., Mauriat, M., Guénin, S., Pelloux, J., Lefebvre, J. F., Louvet, R., Rusterucci, C., Moritz, T., Guerineau, F., Bellini, C. and Van Wuytswinkel, O. (2008). The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. *Plant Biotechnology Journal,* **6(6)**: 609-618.

Haque, T., Faury, D., Albrecht, S., Lopez-Aguilar, E., Hauser, P., Garami, M., Hanzély, Z., Bognár, L., Del Maestro, R. F., Atkinson, J., Nantel, A. and Jabado, N. (2007). Gene expression profiling from formalin-fixed, paraffin-embedded tumors of pediatric glioblastoma. *Clinical Cancer Research,* **13 (21)**: 6284-6292.

Hsiao, L. L., Dangond, F., Yoshida, T., Hong, R., Jensen, R.V., Misra, J., Dillon, W., Lee, K. F., Clark, K.E., Haverty, P., Weng, Z., Mutter, G.L., Frosch, M.P., MacDonald, M. E., Milford, E.L., Crum, C.P., Bueno, R., Pratt, R.E., Mahadevappa, M., Warrington, J. A., Stephanopoulos, G., Stephanopoulos, G. and Gullans, S.R. (2001). A compendium of gene expression in normal human tissues. *Physiological genomics,* **7(2)**: 97-104.

Maier-Hauff, K., Rothe, R., Scholz, R., Gneveckow, U., Wust, P., Thiesen, B., Feussner, A., von Deimling, A., Waldoefner, N., Felix, R. and Jordan, A. (2007). Intracranial thermotherapy using magnetic nanoparticles combined with external beam

radiotherapy: results of a feasibility study on patients with glioblastoma multiforme. *Journal of Neuro-oncology*, **81(1)**: 53-60.

Ramakers, C., Ruijter, J.M., Deprez, R.H. and Moorman, A.F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters*, **339(1)**: 62-66.

Scrideli, C.A., Carlotti, C.G.J., Okamoto, O.K., Andrade, V.S., Cortez, M.A., Motta, F.J., Lucio-Eterovic, A.K., Neder, L., Rosemberg, S., Oba-Shinjo, S.M., Marie, S.K. and Tone, L.G. (2008). Gene expression profile analysis of primary glioblastomas and non-neoplastic brain tissue: identification of potential target genes by oligonucleotide microarray and real-time quantitative PCR. *Journal of Neuro-oncology*, **88(3)**: 281-291.

Sulman, E. P., Guerrero, M. and Aldape, K. (2009). Beyond grade: molecular pathology of malignant gliomas. *Seminars in Radiation Oncology*, **19(3)**: 142-149.

Tricarico, C., Pinzani, P., Bianchi, S., Paglierani, M., Distante, V., Pazzagli, M., Bustin, S. A. and Orlando, C. (2002). Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. *Analytical Biochemistry*, **309(2)**: 293-300.

Warrington, J.A., Nair, A., Mahadevappa, M. and Tsyganskaya, M. (2000). Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. *Physiological Genomics*, **2(3143-147)**.

Watson, J.D., Hopkins, N.H., Roberts, J.W., Steitz, J.A. and Weiner, A.M. (1965). Molecular biology of the gene. Menlo Park, CA: Benjamin/Cummings.

Wong, M.L. and Medrano, J.F. (2005). Real time PCR for mRNA quantitation. *BioTechniques*, **39(1):** 75-85.