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ORIGINAL ARTICLE

Stability of endogenous reference genes in postmortem human brains for normalization of quantitative real-time PCR data: comprehensive evaluation using geNorm, NormFinder, and BestKeeper

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Abstract In forensic molecular pathology, quantitative real-time polymerase chain reaction (RT-qPCR) provides a rapid and sensitive method to investigate functional changes in the death process. Accurate and reliable relative RT-qPCR requires ideal amplification efficiencies of target and reference genes. However, the amplification efficiency, changing during PCR, may be overestimated by the traditional standard curve method. No single gene meets the criteria of an ideal endogenous reference. Therefore, it is necessary to select suitable reference genes for specific requirements. The present study evaluated 32 potential reference genes in the human brain of 15 forensic autopsy cases using three different statistical algorithms, geNorm, NormFinder, and BestKeeper. On RT-qPCR data analyses using a completely objective and noise-resistant algorithm (Real-time PCR Miner), 24 genes met standard efficiency criteria. Validation of their stability and suitability as reference genes using geNorm suggested IPO8 and POLR2A as the most stable ones, and NormFinder indicated that IPO8 and POP4 had the highest expression stabilities, while BestKeeper highlighted ABL1 and ELF1 as reference genes with the least overall variation. Combining these three algorithms suggested the genes IPO8, POLR2A, and PES1 as stable endogenous references in RT-qPCR analysis of human brain samples, with YWHAZ, PPIA, HPRT1, and TBP being the least stable ones. These findings are inconsistent with those of previous studies. Moreover, the relative stability of target and reference genes remains unknown. These observations suggest that suitable reference genes should be selected on the basis of specific requirements, experiment conditions, and the characteristics of target genes in practical applications.

Keywords Quantitative real-time PCR · Endogenous reference genes · geNorm · NormFinder · BestKeeper · Human postmortem brain material

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Introduction

Quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR) is a sensitive, efficient, and reliable molecular technique to determine changes in mRNA expressions [1, 2] and has also been used in the field of forensic sciences, including DNA technology for the criminal justice process as well as postmortem gene expression analysis [3, 4]. RT-qPCR can detect the systemic pathophysiological changes involved in the death process that cannot be detected by morphology [5–8]. This procedure may be useful in investigating

functional alterations in the brain after insults in forensic neuropathology [9].

The most common procedures in RT-qPCR are relative measurements of gene expressions of interest after normalization using endogenous reference gene(s). Accurate and reliable relative RT-qPCR requires ideal amplification efficiencies of target and reference genes [10]; selection of adequate reference genes is essential. Previous studies suggested successful application of conventional reference genes for the myocardium, lungs, kidneys, and skeletal muscle [7, 11–14]. For estimating the amplification efficiency, however, conventional standard curve method is time consuming, requiring the production of repeatable and reliable standards [15, 16]. Furthermore, the amplification efficiency changes during PCR, being relatively stable in the early exponential phase and gradually declining in later cycles [1, 17]; it may be overestimated by the standard curve method [18]. There are several alternative methods for calculating the amplification efficiency on the basis of raw data collected during PCR [17–24], which has been reported to be more accurate than that derived from the standard curve method [1, 18, 25]. Furthermore, expressions of several conventional housekeeping genes have been shown to vary due to nutritional or hormonal factors, biological processes, and/or tissue or cell types; a single housekeeping gene may not meet the criteria of an ideal reference gene [26]. The influence of prolonged agony on RT-qPCR data of the postmortem human brain has also been reported [27].

Since 2002, several statistical algorithms, such as geNorm [28], NormFinder [29], and BestKeeper [30], have been developed to select stably expressed reference genes, and the use of multiple reference genes for accurate normalization in RT-qPCR was proposed. GeNorm determines the expression stability of candidate reference genes by gene stability measure (M), selecting an optimal number of reference genes out of a larger group of candidate genes. NormFinder evaluates the expression stability of each single reference gene and takes into account intra- and intergroup variations for normalization. BestKeeper analyzes variabilities in the expression of candidate reference genes by calculation of cycle threshold (Ct) data variations.

In the present study, amplification efficiencies in RT-qPCR were calculated for 32 potential reference genes in postmortem human brain tissues using a completely objective and noise-resistant algorithm (Real-time PCR Miner) [24] to select candidate reference genes; then, the stability and suitability in RT-qPCR were evaluated using three different statistical algorithms, geNorm, NormFinder, and BestKeeper, to examine the efficacy of candidates for possible practical application in postmortem investigations.

Material and methods

Sample collection

Human brains of forensic autopsy cases (n=15) at our institute, including blunt brain injury (n=12) and sudden cardiac death (SCD, n=3), were examined. A thorough neuropathological analysis was performed as part of our routine investigation and cases with any preexisting neurological pathologies were excluded in the present study. SCD cases included those due to acute ischemic heart disease with or without apparent focal myocardial necrosis (infarction) without any evidence of cause of death other than a cardiac attack [31]. Details are shown in Table 1. Postmortem interval was defined as the estimated time from death to autopsy and survival time was the estimated period from the onset of fatal insult to death; these were estimated on the basis of autopsy findings and circumstantial evidence in autopsy documents. Sample collections and analyses described below were performed within the framework of our routine casework, following the Autopsy Guidelines (2009) and Ethical Guidelines (1997 and 2003) of the Japanese Society of Legal Medicine, approved by our Institutional Ethics Committee.

Brain tissue samples were taken from consistent sites in the parietal lobe of left cerebral hemispheres at autopsy as part of our routine work. In blunt brain injury cases, tissue

Table 1 Case profiles (n=15)

Case number	Age (years)	Gender	ST (hours)	PMI (hours)	A_{260}/A_{280} ratio	RIN
Blunt brain injury						
Case 1	29	F	0.1	13	1.98	5.5
Case 2	54	F	2.5	20	1.86	5.1
Case 3	31	M	3	11	1.97	5.2
Case 4	44	M	6	26	1.99	5.5
Case 5	57	M	6	15	1.94	6.4
Case 6	35	M	12	22	1.86	5.0
Case 7	53	M	20	43	1.87	5.7
Case 8	67	M	36	33	1.93	5.7
Case 9	78	M	72	30	1.93	4.5
Case 10	59	M	192	27	1.96	4.2
Case 11	65	M	312	34	1.96	4.3
Case 12	79	M	384	31	1.81	1.4
SCD						
Case 13	62	F	<0.5	14	2.03	6.3
Case 14	72	M	<0.5	22	1.96	6.9
Case 15	16	M	<0.5	20	2.00	6.7

ST survival time, *PMI* postmortem interval, *RIN* RNA integrity number, *F* female, *M* male, *SCD* sudden cardiac death

specimens distant from primary lesions were selected [32]. All samples were immediately submerged in 1 ml of RNA stabilization solution (RNAlaterTM, Ambion, Austin) and stored at −80 °C until use.

Extraction of total RNA and cDNA synthesis

Total RNA was isolated from 100 mg of sample using ISOGEN (Nippon Gene, Toyama) according to the ISOGEN RNA Extraction Protocol Procedure provided by the manufacturer. After extraction, the RNA concentration was estimated by spectrophotometric analysis using NanoDrop 1000 (Thermo Scientific, Wilmington, USA). cDNA copies of total RNA were obtained using a High Capacity RNA-to-cDNA kit (Applied Biosystems Japan, Ltd.) then were adjusted to a concentration equivalent to 5 ng/μl of total RNA using nuclease-free water.

Evaluation of the quality and integrity of RNA samples

RNA purity was determined using 260/280 absorbance (A_{260}/A_{280}) ratios. The RNA integrity number (RIN) was determined using an RNA 6000 Nano Labchip kit in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) following the manufacturer's protocol.

Reference genes selection

TaqMan Express Human Endogenous Control Plates (Applied Biosystems, Foster City, USA) were used, which contained 32 candidate genes selected from literature searches [33]. Applied Biosystems declared that all these assays have been designed using the same design algorithm and have been extensively tested (details shown in Supplementary Material 1).

RT-qPCR

RT-qPCR reactions were run in 96-well reaction plates with a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA). RT-qPCR was performed with 10 μl cDNA (corresponding to the cDNA reverse transcribed from approximately 50 ng RNA) in 20 μl reaction mix containing 10 μl TaqMan Gene Expression Master Mix (2×) and the above-mentioned TaqMan Gene Expression Assays (lyophiled powder). Thermal cycling conditions included 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 15 s, and 60 °C for 1 min. The threshold cycle (Ct) was calculated by the instrument software automatically (threshold value at 0.2). Raw fluorescent data (normalized reporter values, Rn values) were also exported.

Statistical analysis

Amplification efficiencies were calculated from raw fluorescent data (Rn values), using the Real-time PCR Miner program [24]; the arithmetic mean values of amplification efficiencies were used for further relative quantification. Correlation analyses between pairs of parameters were performed arbitrarily using linear regression (Pearson correlation) with XLSTAT 2012 (Addinsoft, Paris, France).

To compare gene expression stability and rank, geNorm [28], NormFinder [29], and BestKeeper [30] algorithms were used. For geNorm, raw Ct values were transformed to relative non-normalized quantities (Q), according to geNorm manual (http://medgen.ugent.be/~jvdesomp/genorm/geNorm_manual.pdf), with regard to the specific amplification efficiency calculated by Real-time PCR Miner program, by the equation $Q=E^{\Delta \mathrm{Ct}}$, where E is the exponential amplification and ΔCt=min Ct−sample Ct, where min Ct is the lowest Ct value of each gene and sample Ct is the Ct value of the sample being transformed [34, 35]. The highest relative quantities for each gene were set to 1. NormFinder was performed by GenEx Standard software (bioMCC, Freising, Germany), which can indicate the optimal number of reference genes by calculating the Accumulated Standard Deviation (Acc.S.D.). BestKeeper was performed by RefFinder, which is a user-friendly web-based comprehensive tool developed for evaluating and screening reference genes from extensive experimental data sets (www.leonxie.com/referencegene.php). Since RefFinder uses 2 as the default exponential amplification value, Ct values corrected by their respective calculated amplification efficiencies (from Real-time PCR Miner) using GenEx were used as input data. Rank orders of gene stability values from the most stable to least stable ones were calculated by each algorithm. Comprehensive ranking orders of these candidate genes were available from the three methods.

Results

RNA purity and integrity

RNA purity, determined using 260/280 absorbance (A_{260}/A_{280}) ratios, ranged from 1.81 to 2.03. There were no age, survival time, or postmortem interval dependences on Pearson correlation analysis ($p>0.05$) (Table 1).

RIN varied largely among samples, ranging from 1.4 to 6.9, and showed no age or postmortem interval-dependent changes; however, significant survival time-dependent decreases were detected ($R^2=0.72$, $p<0.05$) (Table 1). There was no difference between brain injury and SCD groups when short survival cases (survival time <6 h) were compared, despite a small case number for SCD.

Amplification efficiency

RT-qPCR reactions were performed successfully for all candidate reference genes in each sample with PCR amplification efficiencies (mean values) ranging from 70 % (18S) to 109 % (RPLP0). Calculated amplification efficiencies showed small interindividual variations (standard deviation, SD<5 %) except for CDKN1B (SD=7 %) (Table 2). Eight out of 32 candidate reference genes, 18S, GAPDH, ACTB, CDKN1A, GADD45A, PSMC4, MT-ATP6, and MRPL19, did not meet standard efficiency criteria (90–110 % of efficiency), but another 24 genes met the criteria requirements; these genes were used for further analyses on geNorm, NormFinder, and BestKeeper.

Expression levels of candidate reference genes

Mean Ct values presented a wide range of expression levels. ELF1 showed the least interindividual variation (SD=0.67), while YWHAZ had the largest variation (SD=3.05). The most abundant transcript was 18S with the lowest mean Ct value of 12.11. HMBS showed the least expression with the highest mean Ct value of 29.74, as shown in Table 2.

Relative non-normalized quantity analysis

When relative non-normalized quantities, transformed from raw Ct values, were used, p values, coefficients of determination (R^2), and equation of the model ($y=b0+b1\times x$) could

Table 2 Amplification efficiency and Ct values of candidate reference genes

Gene symbol	Amplification efficiency				Ct value			
	Max	Min	Mean	SD	Max	Min	Mean	SD
18S	0.74	0.64	0.70	0.03	15.48	10.53	12.11	1.19
GAPDH	0.88	0.81	0.83	0.02	27.08	21.34	22.79	1.47
HPRT1	1.04	0.97	1.00	0.02	30.93	23.51	26.30	2.23
GUSB	1.04	0.95	0.99	0.03	30.58	26.48	27.54	1.15
ACTB	0.85	0.77	0.80	0.02	27.91	21.81	23.12	1.53
B2M	1.08	0.97	1.03	0.03	24.72	20.69	22.99	0.88
HMBS	1.10	0.98	1.03	0.03	32.68	28.19	29.74	1.16
IPO8	1.07	0.97	1.02	0.02	30.28	26.95	28.06	0.89
PGK1	1.01	0.94	0.98	0.02	27.41	22.58	24.17	1.32
RPLP0	1.12	1.04	1.09	0.02	26.76	23.78	24.61	0.87
TBP	1.11	1.03	1.06	0.02	33.67	27.38	29.44	1.71
TFRC	1.07	0.99	1.03	0.03	28.96	25.20	26.62	0.99
UBC	1.12	0.99	1.08	0.03	26.63	21.84	23.65	1.25
YWHAZ	1.02	0.93	0.98	0.02	33.27	20.40	29.32	3.05
PPIA	1.05	0.94	0.98	0.03	28.35	20.25	22.28	2.22
POLR2A	1.03	0.97	1.00	0.02	29.06	26.11	27.08	0.90
CASC3	0.99	0.92	0.96	0.02	26.71	24.62	25.59	0.75
CDKN1A	0.93	0.84	0.89	0.03	31.18	25.00	28.46	1.95
CDKN1B	1.26	1.02	1.07	0.07	27.32	23.91	25.29	0.89
GADD45A	0.92	0.88	0.89	0.01	31.15	25.81	28.16	1.36
PUM1	1.01	0.98	1.00	0.01	28.53	24.77	25.98	1.16
PSMC4	0.97	0.83	0.87	0.03	31.36	26.04	27.82	1.40
EIF2B1	1.08	0.97	1.04	0.03	31.24	26.92	28.63	1.18
PES1	0.99	0.92	0.95	0.02	28.64	25.93	26.98	0.75
ABL1	1.00	0.93	0.96	0.02	29.76	27.09	28.10	0.69
ELF1	1.01	0.92	0.97	0.03	29.37	26.83	28.29	0.67
MT-ATP6	0.89	0.80	0.83	0.02	17.62	14.67	15.66	0.86
MRPL19	0.93	0.84	0.89	0.03	29.02	25.76	26.89	0.93
POP4	1.00	0.94	0.96	0.01	30.52	25.87	27.28	1.25
RPL37A	1.01	0.92	0.96	0.03	23.57	19.94	20.85	0.98
RPL30	1.03	0.94	0.97	0.02	26.53	22.44	23.21	1.02
RPS17	0.98	0.91	0.94	0.02	26.11	22.54	23.27	0.93

Ct cycle threshold, *SD* standard deviation

be calculated based on linear regression (Pearson correlation). The relative non-normalized quantities of 21 out of 32 genes showed RIN-dependent increases (R^2=0.32–0.76, $p<0.05$, $b1$=0.1009–0.1652); however, no correlation to post-mortem interval was detected (details shown in Supplementary Material 2). There was no difference in amplicon length between genes with and without correlation of mRNA expression to RIN (n=21 and n=11, respectively; t test, $p>0.05$). Substantial variations of relative non-normalized quantities were detected among these 32 genes on Pearson correlation analysis, ranging from insignificant to very strong correlations (between POLR2A and PUM1, R^2=0.93) (details shown in Supplementary Material 3).

GeNorm analysis

GeNorm ranked the 24 candidate reference genes that met standard efficiency criteria (Fig. 1a). The most stable ones were IPO8 and POLR2A, with an M value of 0.26, followed by PES1 and CDKN1B (M values, 0.30 and 0.31, respectively). The least stable ones were YWHAZ, PPIA, HPRT1, and TBP. Pairwise variation (V) was calculated based on normalization factor values (NF_n and NF_{n+1}) after the inclusion of the least stable reference gene and indicated if the extra reference gene added to the stability of the normalization factor: V2/3 showed a V value of 0.095, below the threshold of 0.15. The V value was the lowest when the 15th most stable gene (RPLPO) was added (V14/15, 0.039), but further addition of other genes increased the V values (Fig. 1b).

NormFinder analysis

Analyzed on NormFinder, the most stable gene was IOP8, followed by POP4, POLR2A, and PUM1, while the least stable ones were YWHAZ, PPIA, HPRT1, and TBP (Fig. 2a). Taking advantage of NormFinder, the Acc.S.D., as an indicator of the optimal number of reference genes

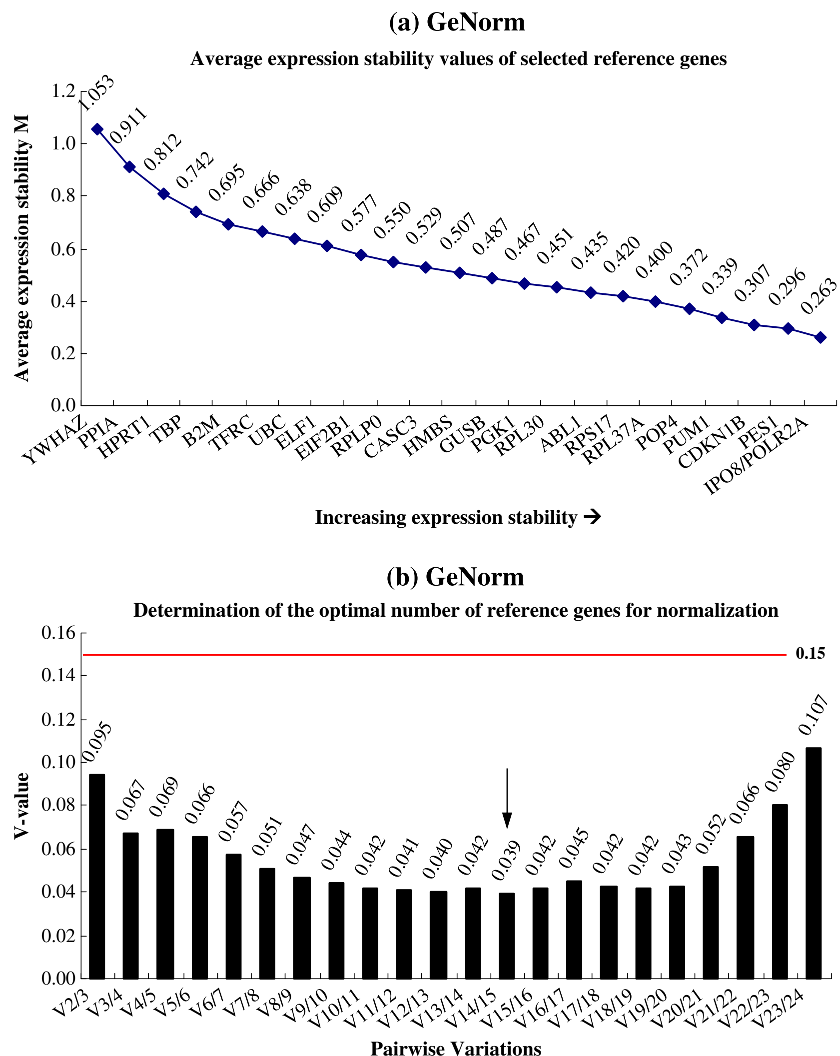

Fig. 1 Average expression stability values (M) and pairwise variation of candidate reference genes using geNorm analysis. **a** Expression stability of genes from the least stable (*left*) to most stable (*right*). **b** Pairwise variation analysis to determine the optimal number of reference genes for normalization

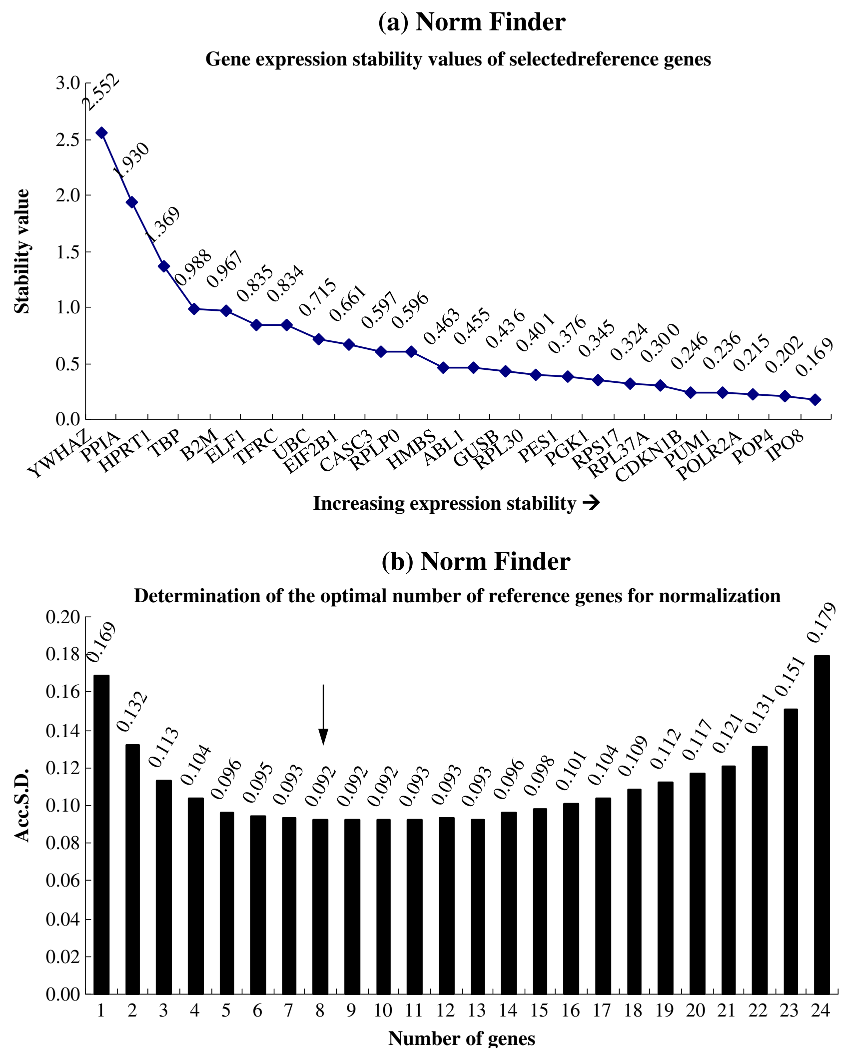

Fig. 2 Gene expression stability values and accumulated standard deviation (Acc.S.D.) analysis using NormFinder. **a** Gene expression stability values of genes from the least stable (*left*) to most stable (*right*). **b** Determination of the optimal number of reference genes on the calculation of the Acc.S.D. by GenEx Standard software

[36], could also be calculated by GenEx Standard software and showed that the Acc.S.D. (0.092) was the lowest when eight reference genes were used (Fig. 2b).

BestKeeper analysis

Analyzed on BestKeeper, the most stable gene that showed the least overall variation was ABL1 (SD=0.516), followed by ELF1, PES1, and B2M, while the least stable ones were YWHAZ and HPRT1, followed by PPIA and TBP (Fig. 3). According to BestKeeper, in addition, 19 out of 24 genes were considered to display stable expressions (Ct value, SD <1.0), while the expressions of YWHAZ, HPRT1, PPIA, TBP, and PGK1 were unstable (Ct value, SD>1.0).

Comprehensive ranking order

Each gene was ranked according to the three methods, respectively, from rank 1 (most stable) to 24 (least stable), the arithmetic mean ranking value of each gene was calculated, and the comprehensive gene stability ranking order could be obtained (Table 3). The three most stable reference genes were IPO8, PES1, and POLR2A, while the least stable reference genes were YWHAZ, PPIA, HPRT1, and TBP. Results were consistent with those of geNorm analysis, only differing in the ranking orders of the most stable genes.

Correlations of relative mRNA quantities among the aforementioned selected genes IPO8, PES1, and POLR2A, analyzed by regression equation analysis, were high and almost equivalent (R^2=0.73–0.84, p<0.05). In addition, GAPDH showed a high correlation with IPO8 (R^2=0.66, p<0.05), while correlations were lower or insignificant for ACTB and B2M (R^2=0.49, p<0.05 and R^2<0.0001, p<0.05, respectively).

Discussion

The accuracy of gene expression analysis using RT-qPCR can be influenced by the quality of RNA; it is preferable to

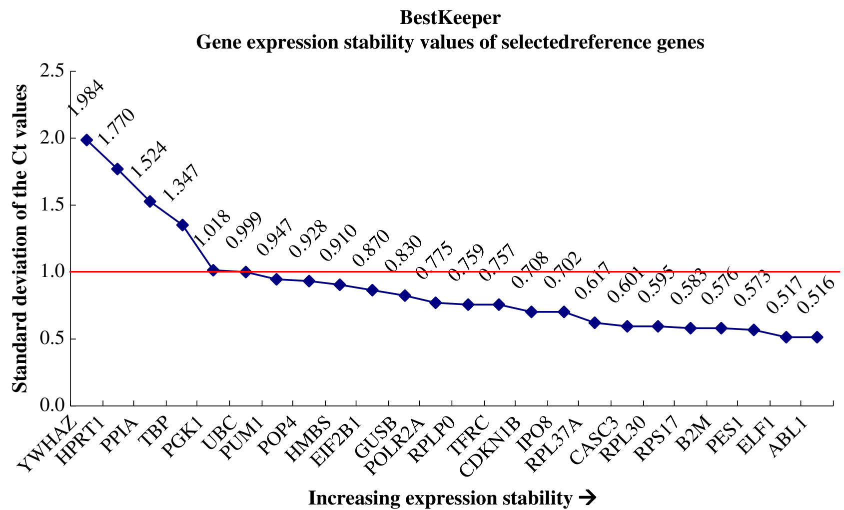

Fig. 3 Gene expression stability values of genes from the least stable (*left*) to most stable (*right*), by calculation of cycle threshold (Ct) data variation, using BestKeeper

use high-quality intact RNA [37]. However, unlike animal experimentation, in which the condition of sample collection can be controlled, RNA degradation is inevitable and unpredictable for human tissues collected at autopsy [38, 39]. In general, the RNA integrity of autopsy material is affected by two major factors, the influence of agony and postmortem interference [27]. In the present study of the human brain, RIN, as an indicator of RNA integrity, showed no postmortem interval-dependent changes in reference gene expressions; however, significant decreases depending on survival time indicated that RNA integrity was more strongly affected by deterioration of the brain during prolonged agony before death. These findings are consistent with those in previous studies, which suggested the influence of brain acidosis on gene transcription and mRNA stability in prolonged death, including those of reference genes [27, 35]. These observations suggest that whole gene expressions, including reference genes, may be deteriorated in prolonged death processes. In such cases, careful evaluation of RT-qPCR data is needed for the interpretation of specific alterations in target gene expressions.

Table 3 Comprehensive ranking order

Ranking order	geNorm	NormFinder	BestKeeper	Comprehensive ranking (mean rank value)
1	POLR2A	IPO8	ABL1	IPO8 (4.00)
2	IPO8	POP4	ELF1	PES1 (5.00)
3	PES1	POLR2A	PES1	POLR2A (5.67)
4	CDKN1B	PUM1	B2M	CDKN1B (6.33)
5	PUM1	CDKN1B	RPS17	RPS17 (6.67)
6	POP4	RPL37A	RPL30	RPL37A (7.00)
7	RPL37A	RPS17	CASC3	ABL1 (7.33)
8	RPS17	PGK1	RPL37A	POP4 (8.33)
9	ABL1	PES1	IPO8	RPL30 (8.67)
10	RPL30	RPL30	CDKN1B	PUM1 (9.00)
11	PGK1	GUSB	TFRC	CASC3 (12.00)
12	GUSB	ABL1	RPLP0	GUSB (12.33)
13	HMBS	HMBS	POLR2A	ELF1 (12.67)
14	CASC3	RPLP0	GUSB	PGK1 (13)
15	RPLP0	CASC3	EIF2B1	RPLP0 (13.67)
16	EIF2B1	EIF2B1	HMBS	HMBS (14.00)
17	ELF1	UBC	POP4	B2M (14.67)
18	UBC	TFRC	PUM1	EIF2B1 (15.67)
19	TFRC	ELF1	UBC	TFRC (16.00)
20	B2M	B2M	PGK1	UBC (18.00)
21	TBP	TBP	TBP	TBP (21.00)
22	HPRT1	HPRT1	PPIA	HPRT1 (22.33)
23	PPIA	PPIA	HPRT1	PPIA (22.67)
24	YWHAZ	YWHAZ	YWHAZ	YWHAZ (24.00)

With regard to the relative non-normalized quantity, large variations were detected among reference genes: 21 out of 32 candidate reference genes showed RIN-dependent increases in the relative non-normalized quantity; however, none of them correlated to postmortem interval. Previous investigation of the effect of RNA integrity, also using RIN, suggested that short amplicons (70–250 bp) had fewer chances to break than longer ones (>400 bp), independent of RIN [37]. In the present study, using a commercial kit containing reference genes with short amplicons (56–187 bp), the correlation between relative non-normalized quantity and RIN were independent of amplicon lengths. Furthermore, genes that showed RIN-dependent relative non-normalized quantities presented only slight tendencies toward increases ($b1=$ 0.1009–0.1652), although the coefficients of determination varied largely (R^2=0.32–0.76). These findings suggest a minor influence of RNA integrity on mRNA quantification that can be reduced to a minimum by means of relative quantification. Previous studies using forensic autopsy

materials also demonstrated that reference genes degraded gradually and in parallel with target genes; relative quantities changed little up to 48 h postmortem [12]. Degradation characteristics in human postmortem tissues still need to be documented in individual studies.

Relative quantification methods assume that the amplification efficiency of the reaction is ideal (100 %) and constant for each sample [1, 40], which can rarely be achieved or verified [24]. Real-time PCR Miner can provide the best precision in estimating the amplification efficiency independent of the PCR instrument [41, 42]. In the present study, most calculated amplification efficiencies showed small interindividual variations; however, 8 out of 32 candidate reference genes did not meet standard efficiency criteria (100±10 % of efficiency) and were excluded in further analyses. It has to be stated that calculated amplification efficiencies were partly different from those estimated by the standard curve method in previous studies [33–35], in which cDNA samples were derived from either a random pool [33, 34] or even a commercial product [35]. Aside from the efficacy of algorithms, comparative studies using different tissue materials are needed.

With the help of geNorm, NormFinder, and BestKeeper, gene expression stabilities of individual reference genes were identified as follows. GeNorm suggested IPO8 and POLR2A as the most stable ones. A threshold V value of 0.15 is recommended as a cutoff value by geNorm to determine the optimal number of reference genes [28]. In the present study, only two reference genes (IPO8 and POLR2A, V2/3=0.0905) were needed to be below the threshold value, although the lowest V value was achieved when the 15th most stable gene (RPLPO) was added. Further addition of genes increased V values, indicating a negative impact on the normalization process. These findings were different from those described in a previous study, which found HMBS, SDHA (succinate dehydrogenase complex, subunit A), and GAPDH as the most stable ones among ten potential reference genes examined [35], suggesting fluctuations owing to analytical conditions, including instruments and reagents.

NormFinder [29], which is less sensitive to co-regulation of reference genes [43], identified that IPO8 and POP4 had the highest expression stabilities, which partly supported the geNorm analysis. This method showed that the optimal number of reference genes was 8, as indicated by the lowest Acc.S.D. (0.092); however, the setting of such a large number of reference genes is not practical [44]. Considering that Acc.S.D. approximated the optimal level when the third most stable gene (POLR2A) was added (Acc.S.D, 0.113), the first three most stable reference genes (IPO8, POP4, and POLR2A) may be used to save on cost and time.

BestKeeper identified ABL1, ELF1, PES1, and B2M as reference genes with the least overall variation, which was different from the results of geNorm and NormFinder analyses mentioned above. Both geNorm and NormFinder use relative quantities transformed from Ct values for stability calculation, while BestKeeper uses Ct values directly. This may explain the different output among these three methods [45]; however, there were only small differences in the SD of Ct values between highest rank genes in BestKeeper (ABL1, ELF1, PES1, and B2M; SD, 0.516–0.576 Ct) and geNorm (POLR2A, IPO8, and PES1; SD 0.573–0.775 Ct) or NormFinder (IPO8, POP4, and POLR2A; SD, 0.702–0.928 Ct), showing stable expressions (SD<1.0 Ct).

To evaluate the results from the three algorithms together, a comprehensive ranking order of each reference gene was calculated: the three most stable reference genes were IPO8, PES1, and POLR2A, while the least stable reference genes were YWHAZ, PPIA, HPRT1, and TBP. These results were consistent with those of geNorm analysis, only differing in the ranking orders of the most stable genes. Therefore, three reference genes, IPO8, PES1, and POLR2A, may be recommended for normalization in postmortem human brain tissues. These three genes showed high and equivalent correlations in the relative non-normalized quantities of respective mRNAs. In addition, one of the conventional reference genes, GAPDH, showed a high correlation with IPO8. This result is consistent with that of a previous study [35]; however, the efficacy of the other conventional references, ACTB and B2M, could not be established. In fact, there is a lack of consensus about the determination of optimal reference genes in postmortem human tissues [35, 36, 46, 47], although most experiments were performed under MIQE Guidelines [48, 49]. The different results may owe to varied requirements and conditions in individual studies. Further programs are needed for standardizing RT-qPCR using postmortem materials.

In conclusion, the present study analyzed 32 potential reference genes in postmortem human brains and identified IPO8, POLR2A, and PES1 as the most suitable references, using three different statistical algorithms, geNorm, NormFinder, and BestKeeper. These findings were partly inconsistent with those of previous studies. Moreover, the relative stability of target and reference genes remains unknown; simultaneous validation of target and reference genes of interest should be considered. These observations suggest that suitable reference genes should be selected on the basis of specific requirements and experiment conditions as well as in consideration of the characteristics of target genes in practical applications.

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References

1. Wong ML, Medrano JF (2005) Real-time PCR for mRNA quantitation. Biotechniques 39(1):75–85
2. Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HR (2005) Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn 5(2):209–219
3. Kim KY, Kwon Y, Bazarragchaa M, Park AJ, Bang H, Lee WB, Lee J, Lee KH, Kim BJ, Kim K (2012) A real-time PCR-based amelogenin Y allele dropout assessment model in gender typing of degraded DNA samples. Int J Leg Med. doi:10.1007/s00414-011-0663-5
4. Maeda H, Zhu BL, Ishikawa T, Michiue T (2010) Forensic molecular pathology of violent deaths. Forensic Sci Int 203(1–3):83–92
5. Maeda H, Ishikawa T, Michiue T (2011) Forensic biochemistry for functional investigation of death: concept and practical application. Leg Med (Tokyo) 13(2):55–67
6. Madea B, Saukko P, Oliva A, Musshoff F (2010) Molecular pathology in forensic medicine—introduction. Forensic Sci Int 203(1–3):3–14
7. Zhao D, Ishikawa T, Quan L, Li DR, Michiue T, Yoshida C, Komatu A, Chen JH, Zhu BL, Maeda H (2008) Tissue-specific differences in mRNA quantification of glucose transporter 1 and vascular endothelial growth factor with special regard to death investigations of fatal injuries. Forensic Sci Int 177(2–3):176–183
8. Wang Q, Ishikawa T, Michiue T, Zhu BL, Guan DW, Maeda H (2012) Intrapulmonary aquaporin-5 expression as a possible biomarker for discriminating smothering and choking from sudden cardiac death: a pilot study. Forensic Sci Int 220(1–3):154–157
9. Iino M, Nakatome M, Ogura Y, Fujimura H, Kuroki H, Inoue H, Ino Y, Fujii T, Terao T, Matoba R (2003) Real-time PCR quantitation of FE65 a beta-amyloid precursor protein-binding protein after traumatic brain injury in rats. Int J Leg Med 117(3):153–159
10. Nolan T, Hands RE, Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. Nat Protoc 1(3):1559–1582
11. Miyazato T, Ishikawa T, Michiue T, Maeda H (2012) Molecular pathology of pulmonary surfactants and cytokines in drowning compared with other asphyxiation and fatal hypothermia. Int J Leg Med 126(4):581–587
12. Zhao D, Zhu BL, Ishikawa T, Quan L, Li DR, Maeda H (2006) Real-time RT-PCR quantitative assays and postmortem degradation profiles of erythropoietin, vascular endothelial growth factor and hypoxia-inducible factor 1 alpha mRNA transcripts in forensic autopsy materials. Leg Med (Tokyo) 8(2):132–136
13. Chen JH, Michiue T, Ishikawa T, Maeda H (2012) Molecular pathology of natriuretic peptides in the myocardium with special regard to fatal intoxication, hypothermia, and hyperthermia. Int J Leg Med 126(5):747–756
14. Wang Q, Ishikawa T, Michiue T, Zhu BL, Guan DW, Maeda H (2012) Molecular pathology of pulmonary edema after injury in forensic autopsy cases. Int J Leg Med. doi:10.1007/s00414-012-0758-7
15. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9):e45
16. Wittwer C, Hahn M, Kaul K (2004) Rapid cycle real-time PCR: methods and applications: quantification. Springer, Berlin
17. Liu W, Saint DA (2002) A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Anal Biochem 302(1):52–59
18. Tichopad A, Dilger M, Schwarz G, Pfaffl MW (2003) Standardized determination of real-time PCR efficiency from a single reaction set-up. Nucleic Acids Res 31(20):e122
19. Liu W, Saint DA (2002) Validation of a quantitative method for real time PCR kinetics. Biochem Biophys Res Commun 294 (2):347–353
20. Harar RP, Kalan A, Kenyon GS (2002) Improving the reproducibility of acoustic rhinometry in the assessment of nasal function. ORL J Otorhinolaryngol Relat Spec 64(1):22–25
21. Ramakers C, Ruijter JM, Deprez RH, Moorman AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett 339(1):62–66
22. Peirson SN, Butler JN, Foster RG (2003) Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. Nucleic Acids Res 31(14):e73
23. Bar T, Stahlberg A, Muszta A, Kubista M (2003) Kinetic Outlier Detection (KOD) in real-time PCR. Nucleic Acids Res 31(17): e105
24. Zhao S, Fernald RD (2005) Comprehensive algorithm for quantitative real-time polymerase chain reaction. J Comput Biol 12 (8):1047–1064
25. Marino JH, Cook P, Miller KS (2003) Accurate and statistically verified quantification of relative mRNA abundances using SYBR Green I and real-time RT-PCR. J Immunol Methods 283(1–2):291–306
26. de Jonge HJ, Fehrmann RS, de Bont ES, Hofstra RM, Gerbens F, Kamps WA, de Vries EG, van der Zee AG, te Meerman GJ, ter Elst A (2007) Evidence based selection of housekeeping genes. PLoS One 2(9):e898
27. Tomita H, Vawter MP, Walsh DM, Evans SJ, Choudary PV, Li J, Overman KM, Atz ME, Myers RM, Jones EG, Watson SJ, Akil H, Bunney WE Jr (2004) Effect of agonal and postmortem factors on gene expression profile: quality control in microarray analyses of postmortem human brain. Biol Psychiatry 55(4):346–352
28. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3(7):RESEARCH0034
29. Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64(15):5245–5250
30. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. Biotechnol Lett 26(6):509–515
31. Zipes DP, Wellens HJ (1998) Sudden cardiac death. Circulation 98 (21):2334–2351
32. Wang Q, Ishikawa T, Michiue T, Zhu BL, Guan DW, Maeda H (2012) Quantitative immunohistochemical analysis of human brain basic fibroblast growth factor, glial fibrillary acidic protein and single-stranded DNA expressions following traumatic brain injury. Forensic Sci Int 221(1–3):142–151
33. Pfister C, Tatabiga MS, Roser F (2011) Selection of suitable reference genes for quantitative real-time polymerase chain reaction in human meningiomas and arachnoidea. BMC Res Notes 4:275
34. Coulson DT, Brockbank S, Quinn JG, Murphy S, Ravid R, Irvine GB, Johnston JA (2008) Identification of valid reference genes for the normalization of RT qPCR gene expression data in human brain tissue. BMC Mol Biol 9:46
35. Koppelkamm A, Vennemann B, Fracasso T, Lutz-Bonengel S, Schmidt U, Heinrich M (2010) Validation of adequate endogenous reference genes for the normalisation of qPCR gene expression data in human post mortem tissue. Int J Leg Med 124(5):371–380
36. Penna I, Vella S, Gigoni A, Russo C, Cancedda R, Pagano A (2011) Selection of candidate housekeeping genes for normalization in human postmortem brain samples. Int J Mol Sci 12 (9):5461–5470
37. Fleige S, Pfaffl MW (2006) RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med 27(2–3):126–139

38. Koppelkamm A, Vennemann B, Lutz-Bonengel S, Fracasso T, Vennemann M (2011) RNA integrity in post-mortem samples: influencing parameters and implications on RT-qPCR assays. Int J Leg Med 125(4):573–580
39. Stan AD, Ghose S, Gao XM, Roberts RC, Lewis-Amezcua K, Hatanpaa KJ, Tamminga CA (2006) Human postmortem tissue: what quality markers matter? Brain Res 1123(1):1–11
40. Gibson UE, Heid CA, Williams PM (1996) A novel method for real time quantitative RT-PCR. Genome Res 6(10):995–1001
41. Arikawa E, Yang J (2007) Comparing algorithms for calculating amplification efficiencies of real-time PCR. Cambridge Healthtech Institute's Third Annual Quantitative PCR, Microarrays, and Biological Validation: Capturing the Complete Biological Story, Providence
42. Graeber K, Linkies A, Wood AT, Leubner-Metzger G (2011) A guideline to family-wide comparative state-of-the-art quantitative RT-PCR analysis exemplified with a Brassicaceae cross-species seed germination case study. Plant Cell 23(6):2045–2063
43. Maroufi A, Van Bockstaele E, De Loose M (2010) Validation of reference genes for gene expression analysis in chicory (*Cichorium intybus*) using quantitative real-time PCR. BMC Mol Biol 11:15
44. Condori J, Nopo-Olazabal C, Medrano G, Medina-Bolivar F (2011) Selection of reference genes for qPCR in hairy root cultures of peanut. BMC Res Notes 4:392
45. Scharlaken B, de Graaf DC, Goossens K, Brunain M, Peelman LJ, Jacobs FJ (2008) Reference gene selection for insect expression studies using quantitative real-time PCR: the head of the honeybee, *Apis mellifera*, after a bacterial challenge. J Insect Sci 8:33
46. Weickert CS, Sheedy D, Rothmond DA, Dedova I, Fung S, Garrick T, Wong J, Harding AJ, Sivagnanansundaram S, Hunt C, Duncan C, Sundqvist N, Tsai SY, Anand J, Draganic D, Harper C (2010) Selection of reference gene expression in a schizophrenia brain cohort. Aust N Z J Psychiatry 44(1):59–70
47. Tunbridge EM, Eastwood SL, Harrison PJ (2011) Changed relative to what? Housekeeping genes and normalization strategies in human brain gene expression studies. Biol Psychiatry 69(2):173–179
48. Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Kibenge FS, Olsvik PA, Penning LC, Toegel S (2010) MIQE precis: practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. BMC Mol Biol 11:74
49. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55(4):611–622