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ORIGINAL ARTICLE

Validation of adequate endogenous reference genes for the normalisation of qPCR gene expression data in human post mortem tissue

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Abstract Gene expression analyses based on messenger RNA (mRNA) profiling require accurate data normalisation. When using endogenous reference genes, these have to be validated carefully. Therefore, we examined the transcript stability of 10 potential reference genes using quantitative real-time polymerase chain reaction: beta actin, 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase, TATA box-binding protein, hypoxanthine phosphoribosyltransferase I, beta-2-microglobulin, hydroxymethylbilane synthase, succinate dehydrogenase complex, subunit A, cyclophilin A and ubiquitin C. The aim of the current study was to assess which reference genes show stable mRNA levels in human post mortem cardiac muscle, skeletal muscle and brain tissue. Considering cardiac muscle tissue, CYCA and TBP were identified as the most stable while in skeletal muscle tissue, SDHA and TBP, and in brain tissue, SDHA and HMBS turned out to be the most stable. Furthermore, we recommend a minimum of four carefully validated endogenous control genes for reliable data normalisation in human post mortem tissue. Parameters influencing the stability of transcript amounts were found to be mainly the post mortem interval in cardiac muscle and skeletal muscle tissue and the donor's cause of death in skeletal muscle and brain samples. Further parameters like gender, age at death and body mass index were found to influence mRNA quantities in skeletal muscle only. The set of stable control genes identified in this study may be used in further studies if the composition of the samples is similar to the one used here.

Keywords RNA · Gene expression · Data normalisation · RT-qPCR · Post mortem tissue

Dedicated to Prof. Stefan Pollak on the occasion of his 60th birthday

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Introduction

Most of the gene expression studies for the investigation of human diseases are based on the use of animal models [1, 2], where changes in expression patterns depend on varying experimental conditions, but recent developments in biomedical research show that the transfer of these data on the human organism has limitations [3].

Consequently, in recent years, interest in human tissue as the target for gene expression studies has clearly increased (e.g. [4, 5]). This trend is reflected in the implementation of human tissue banks [6–8]. Potential material for the tissue banks might be surgical specimens and, to a larger extend, samples obtained during post mortem examinations [4].

During post mortem examination, not only pathologically altered tissue samples but also healthy human control tissue

can be collected in sufficient amounts for extraction of messenger RNA (mRNA). In a previous study, we were able to show the possibility of extracting RNA from post mortem tissue samples obtained during forensic autopsy [9], which is in accordance with several other recent studies [10–13]. While RNA profiling within the forensic community is currently focused on the validation of markers for the identification of body fluids in crime scene stains (e.g. [14–19]), the aim of our work was to figure out whether or not reliable quantitative expression data can be obtained from human post mortem internal organs. Current studies raise hope that in the future, gene expression analysis using post mortem tissue may improve the understanding of the pathophysiology of disease [20–23] and may complement the current methods for the determination of cause and circumstance of death [24].

The method of choice for accurate mRNA profiling is reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR) because of its high sensitivity and the broad range of commercially available assays for numerous transcripts in human genomes [25]. On the other hand, the exceeding quantitative accuracy may cause problems in data interpretation because slight changes in mRNA levels caused by sample processing might be mistaken as changes in gene expression activity. Therefore, correct data normalisation is considered to be crucial [26]. The most widespread method for data normalisation is the use of endogenous reference genes. An ideal reference gene should show stable transcript levels in different tissue types and individuals and should be unaffected in its expression by experimental conditions and disease state or (in case of post mortem tissue) the cause of death, the post mortem interval (PMI) as well as further individual parameters. Opposing the original thought of ubiquitous expression of such genes, several studies already found an existing variation in the expression of commonly used reference genes (e.g. [27, 28]). This leads to the awareness that reference genes have to be validated carefully and that usually more than one, but rather two or more very well validated genes are necessary for correct data normalisation [26, 29].

However, when working with post mortem human tissue, the environmental conditions that the body has been exposed to prior to sample collection cannot be influenced [30]. In this context, one has to deal with a set of potentially changing conditions. Therefore, rather than validating endogenous control genes for one particular study, it seems more reasonable to investigate the expression stability of commonly used endogenous control genes in a larger set of samples to identify the genes with the most stable transcript amounts in post mortem tissue.

The aim of the present study was to identify suitable genes for data normalisation in future gene expression studies and to investigate the parameters influencing the stability of 10 commonly used reference genes in human post mortem cardiac muscle, skeletal muscle and brain tissue. Additionally, the minimum number of genes required for reliable data normalisation was to be determined.

Materials and methods

Tissue samples

A total of 111 human tissue samples from 37 individuals were collected during routine forensic autopsy (vote 272/05 of the ethical committee of the Freiburg University). Three tissue types without obvious pathological changes were used in this study: human cardiac muscle (left ventricle, posterior myocardial wall), human skeletal muscle (M. iliopsoas) and human brain (frontal lobe). Only individuals with a PMI of less than 50 h and without macroscopic signs of putrefaction were considered for sampling. Table 1 gives an overview of PMI, cause of death, gender, age at death and body mass index (BMI). Samples were collected in RNAlater (Ambion, Austin, TX, USA) and transferred to −80°C for long-time storage.

Endogenous reference genes

Ten potential reference genes were chosen according to their common use as endogenous control genes in the

Table 1 Overview of the individuals included in the present study

Parameter		Number	Mean (range)
PMI (h)	<10	9	20.24 (4–42)
	11–20	13	
	21–30	8	
	31–45	7	
Cause of death	Central dysregulation	10	
	Sudden cardiac death	8	
	Asphyxia	9	
	Other[a]	10	
Gender	Male	23	
	Female	14	
Age at death (years)	Under 30	7	46.27 (12–87)
	31–50	16	
	Over 50	14	
BMI (body mass index)	<25	21	24.99 (16.4–37.7)
	>25	16	

The influences of PMI, cause of death, gender, age at death and BMI on the expression level of several genes of interest were addressed

[a] Including five cases of fatal haemorrhage and one case of each: pneumonia, haemopericardium, alcohol intoxication, fire death (47% COHb), head injury/aspiration

literature (Table 2). Genes that belong to different molecular pathways were used to minimise the risk of co-regulation [29].

Extraction of total RNA

To minimise the risk of RNA degradation by means of RNases during the experimental process, all materials and working surfaces were cleaned using RNase Away (Molecular Bioproducts Inc., San Diego, CA, USA) prior to handling the samples. Total RNA was isolated from 200 mg of tissue using RNagents Total Isolation System (Promega, Mannheim, Germany) according to the manufacturer's recommendations. Samples were homogenised in denaturing solution with an IKA-T10 homogenizer (IKA, Staufen, Germany) and finally dissolved in 100 μL nuclease free water. To remove residual genomic DNA, isolated RNA was treated with Turbo DNA-free kit (Ambion, Austin, TX, USA) and purified using RNeasy Mini kit (Qiagen, Hilden, Germany).

Quality control of total RNA

Extraction yield and purity were assessed by spectrophotometric analysis using NanoDrop ND-1000 (PeqLab, Erlangen, Germany). RNA integrity and level of degradation was assessed by on-chip electrophoresis using RNA 6000 Nano kit and Bio-Analyzer 2100 (Agilent Technologies, Böblingen, Germany). Based on the electropherogram, an RNA Integrity Number (RIN) between 1 (completely degraded) and 10 (completely intact) is calculated, taking into account the 18S/28S rRNA peaks as well as the background and possible degradation products [31].

RT-qPCR

RT-qPCR was performed using a two-step protocol. For synthesis of complementary DNA (cDNA), the High-Capacity cDNA Reverse Transcription kit with random hexamer primers and Multi Scribe Reverse Transcriptase (Applied Biosystems, Darmstadt, Germany) was used. cDNA was diluted with HPLC grade water (VWR International, Fontenay sous Bois, France) to a concentration equivalent to 5 ng/μL of total RNA.

Real-time PCR amplification mixture contained 5 μL TaqMan Universal PCR Mastermix and 0.5 μL TaqMan Gene Expression Assays (both Applied Biosystems) for the following genes: beta actin (ACTB), 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TATA box-binding protein (TBP), hypoxanthine phosphoribosyl-transferase I (HPRTI), beta-2-microglobulin (B2M), hydroxymethylbilane synthase (HMBS), succinate dehydrogenase complex, subunit A (SDHA), cyclophilin A (CYCA) and ubiquitin C (UBC; assay IDs are given in Table 2). Template cDNA (2 μL) and 2.5 μL H_2O were added to a total volume of 10 μL. Samples were amplified and detected using a 7500 Sequence Detection System (Applied Biosystems). Reactions were prepared in duplicates for each sample and each of the 10 assays. Copies of the particular endogenous control mRNA were quantified and presented as cycle threshold (C_t) value. Each run contained a no template control as well as a positive control. For the latter, a cDNA synthesised from a commercially available total RNA from human cardiac muscle tissue (Stratagene, Heidelberg, Germany) was used to control possible plate-to-plate differences. For each assay used, a threefold serial dilution of the positive control was used to

Table 2 Internal control genes examined in this study

Gene Symbol	Gene Name	Accession number	Biological function	Assay ID[a]
ACTB	Beta actin	[RefSeq:NM001101.3]	Cytoskeletal structure	Hs00357333_g1
18S rRNA	18S rRNA	[GenBaknk:X03205.1]	Ribosomal formation	Hs99999901_s1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	[RefSeq:NM002046.3]	Involved in glycolysis	Hs00266705_g1
TBP	TATA box-binding protein	[RefSeq:NM003194.3]	Transcription factor	Hs00427620_m1
HPRT1	Hypoxanthine phosphoribosyl-transferase I	[RefSeq:NM00194.1]	Involved in salvage pathway	Hs99999909_m1
B2M	Beta-2-microglobulin	[RefSeq:NM004048.2]	Protein binding (immune response)	Hs00187842_m1
HMBS	Hydroxymethylbilane synthase	[RefSeq:NM000190.3]	Involved in porphyrine metabolism	Hs00609297_m1
SDHA	Succinate dehydrogenase complex, subunit A	[RefSeq:NP004159.2]	Electron transport	Hs00188166_m1
CYCA	Cyclophilin A	[RefSeq:NM021130.3]	Involved in signal transduction	Hs99999904_m1
UBC	Ubiquitin C	[RefSeq:NM021009.4]	Involved in protein degradation	Hs00824723_m1

The genes were chosen to belong to different physiological pathways to reduce the risk of co-regulation. TaqMan real-time PCR assays were chosen to span at least one exon–exon boundary and can be purchased with the given assay IDs (Applied Biosystems, Darmstadt, Germany)

[a] Purchased from Applied Biosystems

create a standard curve and to calculate the amplification efficiency E using the equation $E = 10^{(-1/\text{slope})}$ [25, 32].

Data analysis

Data analysis was performed in two steps: Firstly, the genes with the most stable transcription level in the given set of samples and the minimum number of genes needed for reliable data normalisation were assessed. Secondly, the most stable genes were used for data normalisation of the remaining control genes to analyse the influence of five different parameters on the variability of transcript amounts. For this purpose, samples were assorted according to the following parameters: PMI, cause of death, gender, age at death and BMI. For PMI, samples were assorted to result in evenly distributed groups spanning 10-h intervals. For BMI, samples were assorted according to the WHO standards defining BMI>25 as pre-obesity or obesity, respectively.

For the first step, mean C_t values were used for calculation of ΔC_t values. The exponential function $E^{-\Delta Ct}$ determined the relative, non-normalised quantity of each transcript with regard to the specific amplification efficiency E. To identify the control genes with the most stable amount of transcripts in the given set of samples and to quantify the variation of the 10 endogenous control genes, relative, non-normalised quantities were imported into the geNorm VBA applet [29]. geNorm calculates the gene expression stability measure M for a reference gene as the average pairwise variation V for that gene with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allows ranking of the tested genes according to their expression stability (geNorm manual: http://medgen.ugent.be/~jvdesomp/genorm/geNorm_manual.pdf).

Since the authors recommend the use of at least three reference genes for normalisation, a normalisation factor (NF) is calculated for the three most stable genes by calculating the geometric mean of these genes (NF_n, $n=3$). To determine whether further genes have an impact on NF, a stepwise inclusion of genes with lower stability is performed until the $(n+1)$th gene has no significant impact on the new normalisation factor (NF_{n+1}). To assess the possible need for more than three genes, the pairwise variation ($V_{n/n+1}$) between two sequential normalisation factors (NF_n and NF_{n+1}) is calculated for all samples investigated [29].

For the second step, quantitative mRNA data of the rather unstable genes were normalised against a normalisation factor calculated as the geometric mean of the most stable genes. Therefore, the C_t values of the sample duplicates were imported into the MSExcel VBA applet qBase [33]. The programme employs a delta-C_t relative quantification model with PCR efficiency correction and multiple reference gene normalisation. Some of the algorithms are identical to those used in the geNorm applet. Non-normalised relative quantities are divided by the normalisation factor and calibrated to their mean to generate calibrated normalised relative quantities (CNRQ). Samples were grouped according to the five parameters listed above, and qBase analyses were performed for each parameter and each tissue type independently.

Statistical analysis of CNRQ values applying Box–Whisker plots and Mann–Whitney U test (SPSS for Windows vs. 15.0) yielded information about the possible influence of the five parameters on the quantities of transcripts of each gene.

Results

RNA extraction and integrity

Total RNA was successfully extracted from all 111 samples. The highest mean RNA yield was obtained from cardiac muscle tissue (mean 191.88 ng/mg of tissue, range 21.86–364.23 ng/mg) compared to brain (mean 127.33 ng/mg of tissue, range 8.65–483.12 ng/mg) and skeletal muscle tissue (mean 107.68 ng/mg of tissue, range 10.98–305.8 ng/mg). A correlation between total RNA yield and cause of death, PMI or age at death was not observed. After additional purification, the 260/280 ratios of all samples ranged from 2.0 to 2.1. The RIN was found to be lower in brain (mean 2.9, range 1.4–6.2) than in cardiac muscle (mean 3.9, range 2.1–6.8) and skeletal muscle (mean 4.3, range 1.6–6.9).

RT-qPCR: efficiency, accuracy and plate-to-plate variation

RT-qPCR reactions were performed successfully for all 111 samples and 10 different assays with PCR efficiencies ranging from 87% (SDHA) to 110% (ACTB). All samples used for efficiency estimation showed a very good correlation of duplicates with $r^2>0.98$ (Online Resources 1 in the Electronic Supplementary Material). Since three reaction plates were necessary for each gene—one for each tissue type—a plate-to-plate control sample was added in duplicates in each run. The standard deviation of the C_t values was calculated to estimate inter-run variations and was found to be below or only slightly higher in samples on different plates compared to duplicates on one plate (see Online Resources 2 in the Electronic Supplementary Material).

Stability of endogenous control genes

Stability measures M and pairwise variations obtained by geNorm are given in Fig. 1. In post mortem cardiac muscle tissue (Fig. 1a), the genes with the most stable mRNA

levels were found to be cyclophilin A (CYCA) and TBP, followed by HMBS and SDHA. These four genes represent the minimal number of genes required for reliable data normalisation when using a cut-off value of 0.2 (Fig. 1b). In post mortem skeletal muscle tissue, the four genes required for normalisation were SDHA, TBP, HPRT1 and CYCA (Fig. 1c, d). In post mortem brain tissue, the genes with the most stable post mortem mRNA levels were HMBS, SDHA, GAPDH and UBC (Fig. 1e and f). In all tissues investigated, the very commonly used reference genes 18S rRNA and ACTB showed particularly high *M* values. The transcripts of GAPDH and HPRT1 showed tissue-specific stability. GAPDH revealed high *M* values in skeletal and cardiac muscle but not in brain tissue, and HPRT1 revealed high *M* values in cardiac muscle and brain but not in skeletal muscle.

For GAPDH, another common reference gene, this is also true for skeletal muscle and cardiac muscle, but not for brain tissue.

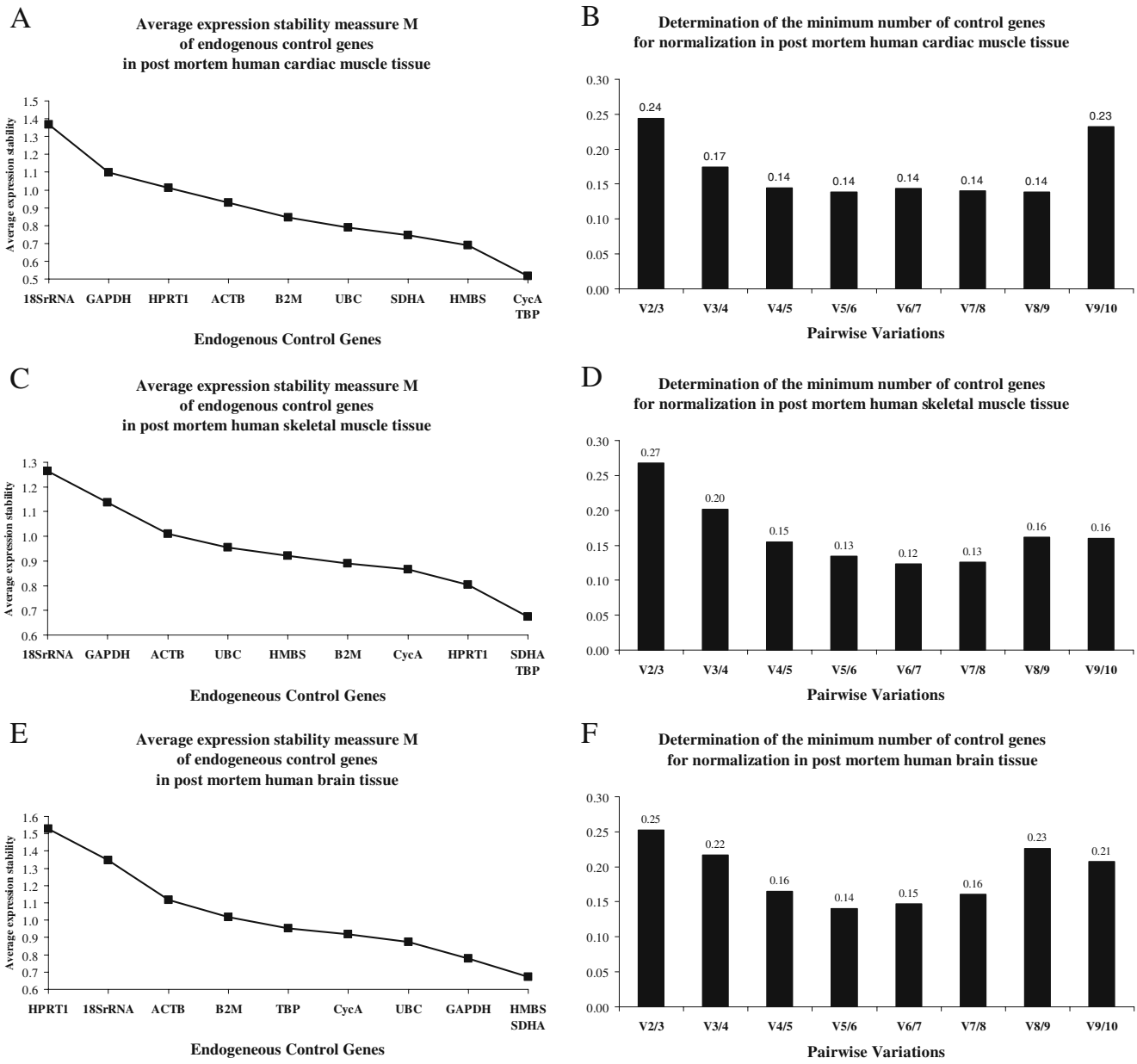


Fig. 1 Example of data analysis. Average expression stability of all genes analysed as well as the determination of the minimal number of control genes needed for data normalisation in cardiac muscle (**a**, **b**), skeletal muscle (**c**, **d**) and brain tissue (**e**, **f**). The minimal number of genes is determined by calculating the pairwise variation of two sequential normalisation factors and stepwise inclusion of additional genes ($V_{n/n+1}$)

Determination of the minimum number of endogenous control genes

In Fig. 1b, d, f, the pairwise variations ($V_{n/n+1}$) are given after stepwise inclusion of genes with higher M values. Vandesompele et al. [29] recommend the use of a guidance level of V=0.15 to determine the minimal number of genes required for reliable data normalisation. The number of genes necessary to obtain V<0.15 are at least five (in cardiac muscle and skeletal muscle tissue) while in brain, six reference genes are necessary to achieve a V value below 0.15.

Parameters influencing transcript stability

To identify parameters that might influence the transcript stability of genes that were found to show rather high M values, the relative transcript quantities observed for these genes were normalised using the suitable reference genes identified by geNorm. Thus, in cardiac muscle tissue, relative quantities of the genes 18S rRNA, GAPDH, B2M and UBC were normalised using a normalisation factor calculated as the geometric mean of the quantities observed for the genes CYCA, TBP, HMBS and SDHA for each sample. Accordingly, the rather unstable genes in skeletal muscle and brain tissue were normalised using normalisation factors calculated from the four most stable genes in each tissue, respectively.

Influence of the post mortem interval

To investigate the influence of post mortem interval on the stability of transcripts, the differences between samples grouped into four defined PMI-stages were analysed (Fig. 2a). In skeletal muscle, GAPDH showed a significantly higher transcript amount in the group “11–20 h” compared to “0–10 h” and “21–30 h” while analysis of UBC revealed significantly lower transcript amounts in the group “21–30 h” compared to samples with shorter or longer PMIs. In cardiac muscle tissue, B2M transcript amounts were significantly lower in short PMI groups than in long PMI groups, and GAPDH transcript quantities showed a trend towards lower amounts in groups with higher PMI. No significant differences were found in brain tissue.

Influence of cause of death

Out of the 37 individuals in this study, 10 died of central dysregulation (cerebral pathology due to trauma or natural cause), eight individuals died of sudden cardiac death, nine died of asphyxia, and 10 died of other various causes of death (Table 1). In skeletal muscle tissue, the transcript level of B2M appeared to be elevated in samples from individuals deceased of cardiac death (Fig. 2b). GAPDH transcript amounts were found to be higher in samples from individuals that died of asphyxia than those that died of central dysregulation. In brain tissue, the mRNA level of B2M, HPRT1 and TBP turned out to be significantly higher in individuals that died of central dysregulation compared to other causes of death. In cardiac muscle tissue, no significant differences of transcript levels were found.

Fig. 2 Analysis of the gene expression level depending on several parameters. Normalisation against identified stable reference genes and statistical analysis identified significant coherencies between the expression level of tissue-depending genes of interest and defined parameters (PMI, cause of death, gender, age at death and BMI). Significances are marked with a *filled diamond*, meaning p<0.05, and *open diamond*, meaning p<0.01, respectively ▶

Influence of gender

This study involved 14 female and 23 male individuals (Table 1). The only statistically significant difference was found in the mRNA amount of UBC showing a higher transcript level in female than in male skeletal muscle (Fig. 2c).

Influence of age at death

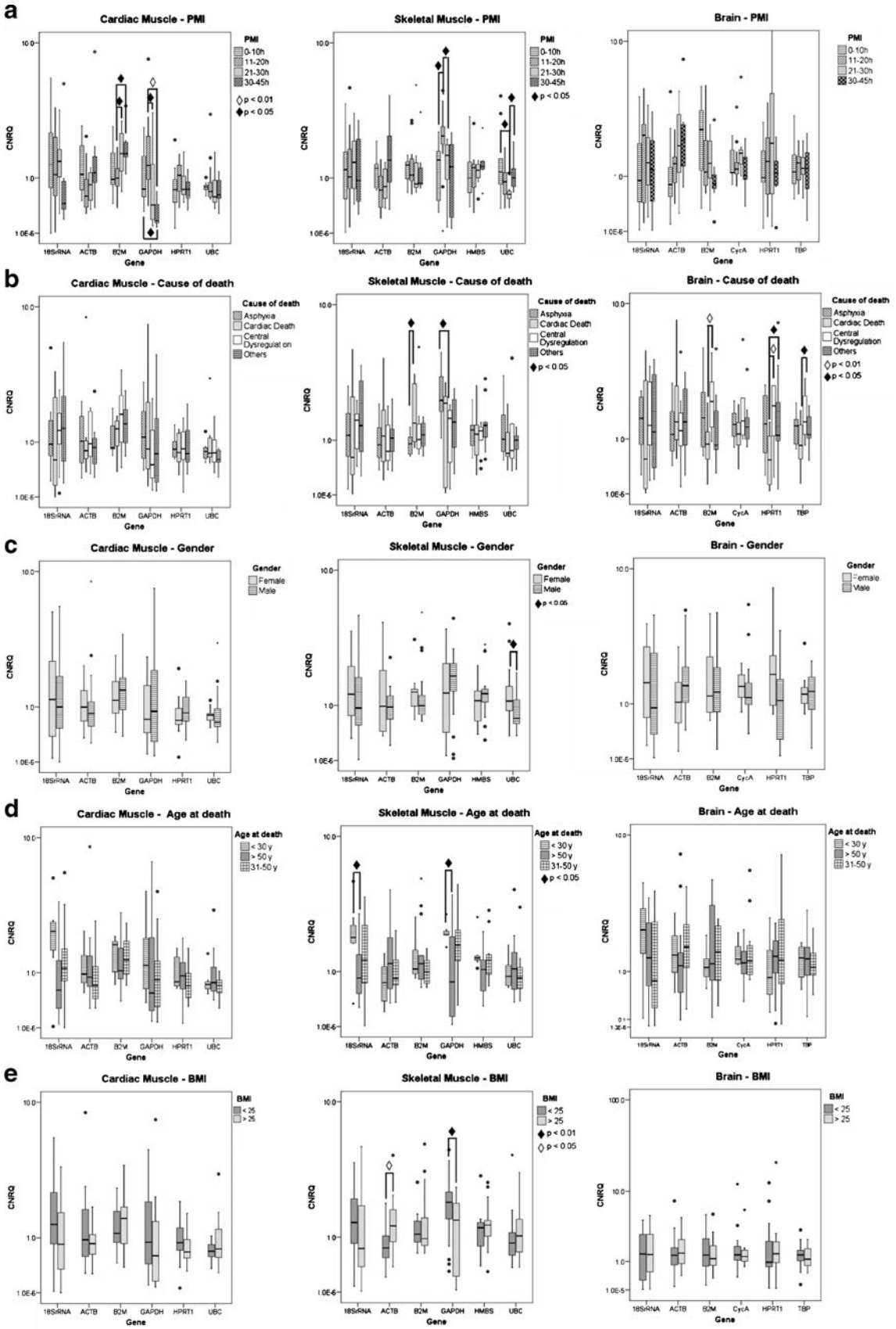
Out of 37 individuals of this study, seven deceased before reaching the age of 30 years, and 16 were aged between 31 and 50. Further, 14 individuals achieved higher ages and were summarised in a group termed “over 50 years” (Table 1). GAPH and 18S rRNA transcripts were found in a higher amount in skeletal muscle of individuals below 30 years of age compared to older individuals. No significant coherency was found in cardiac muscle and brain tissue (Fig. 2d).

Influence of body mass index

Sixteen out of 37 individuals showed a body mass index above 25 (Table 1). The only significant differences between samples from normal-weighted individuals (BMI < 25) and individuals with elevated BMI were observed in skeletal muscle tissue. ACTB mRNA was found in significantly higher and GAPDH mRNA in significantly lower amounts in individuals with BMI > 25 (Fig. 2e).

Discussion

The issue of correct normalisation of quantitative gene expression data is a crucial point in achieving reliable and


a
Cardiac Muscle - PMI
Skeletal Muscle - PMI
Brain - PMI
PMI
0-10h
11-20h
21-30h
30-45h
p < 0.01
p < 0.05
CNRQ
Gene
18SrRNA ACTB B2M GAPDH HPRT1 UBC
18SrRNA ACTB B2M GAPDH HMBS UBC
18SrRNA ACTB B2M CycA HPRT1 TBP
b
Cardiac Muscle - Cause of death
Skeletal Muscle - Cause of death
Brain - Cause of death
Cause of death
Asphyxia
Cardiac Death
Central Dysregulation
Others
p < 0.01
p < 0.05
c
Cardiac Muscle - Gender
Skeletal Muscle - Gender
Brain - Gender
Gender
Female
Male
d
Cardiac Muscle - Age at death
Skeletal Muscle - Age at death
Brain - Age at death
Age at death
< 30 y
> 50 y
31-50 y
e
Cardiac Muscle - BMI
Skeletal Muscle - BMI
Brain - BMI
BMI
< 25
> 25

biologically meaningful results [26]. Data normalisation is important to correct for sample-to-sample differences due to slight differences in samples' sources and integrity, handling and efficiency of the analysis [32], but the quality of quantitative gene expression data can only ever be as good as the normaliser is. Changes in the normaliser will lead to erroneous data interpretation because they might falsely hint at changes in the expression levels of genes of interest or might obscure real differences [34]. In recent studies, the suitability of GAPDH [35], ACTB and 18S rRNA [35–40], as well as CYCA [41] and B2M [37, 42] as endogenous reference genes, has often been analysed, and the stability of these genes was found to be a variable. Therefore, we included these commonly used endogenous reference genes in a set of 10 genes analysed in this study.

With the help of the freely available Excel applet geNorm, we were able to identify the genes with the most stable amount of transcript among a set of 10 candidate reference genes (Table 2). In our set of samples comprising individuals with variable causes of death, post mortem intervals and individual parameters (Table 1), the reference gene SDHA was found to be among the four genes with the most stable amount of mRNA in all tissue types investigated. SDHA codes for a flavoprotein, succinate dehydrogenase, which is involved in the electron transport in the mitochondrial respiratory chain [43]. On the other hand, our results imply that the commonly used endogenous control genes 18S rRNA and ACTB [44] show rather unstable transcript levels in post mortem cardiac muscle, skeletal muscle and brain tissues so that they are not recommendable for normalisation in gene expression studies based on similar sample composition. For post mortem human brain tissue, these findings are in accordance with the findings of Coulson et al. [21]. Varying stabilities of potential endogenous reference genes were recently described by several groups. For example, expression of GAPDH and ACTB was found to be significantly regulated in various experimental conditions and variable between different tissues [45]. In our study, we found that transcript stability seems to be tissue dependent, since different genes were found to be stable in the three tissues investigated, respectively. In fact, the transcripts of GAPDH and HPRT1 showed varying stabilities between different tissues. Thus, it might be interesting to include them in a future gene expression study to investigate whether or not this finding can be explained by tissue-specific metabolic processes. Knowing that, the use of a single gene for data normalisation is not acceptable anymore. Rather, a set of carefully validated reference genes should be used for correct data normalisation [29].

Information about the minimum number of endogenous control genes that are necessary for data normalisation could be obtained from the pairwise variation *V*. The cut-off value, below which additional endogenous control genes have no further impact on correct data normalisation, was recommended as 0.15 [29]. In our set of samples, this would mean that in cardiac muscle and skeletal muscle tissue, five endogenous reference genes would be necessary while in brain tissue, even six genes would be needed to obtain $V < 0.15$. The estimation of the suitable number of control genes has to balance between practical considerations and the pursuit of accuracy. With limited amount of starting material, it might not be practical to use six reference genes to normalise quantitative data from one gene of interest. Therefore, the authors of geNorm also state that this cut-off value of 0.15 should not be taken too strict because the use of the three most stable genes for normalisation is still preferable to many other normalisation strategies (geNorm manual: http://medgen.ugent.be/~jvdesomp/genorm/geNorm_manual.pdf). Since in our sample set we observed rather high values for the pairwise variations of normalisation factors, we recommend the use of at least four very well validated genes for normalisation of quantitative expression data in post mortem tissues.

After normalisation against the stable reference genes, this study examined the impact of different parameters (PMI, cause of death, gender, age at death and BMI) on the transcript level of the remaining six instable genes.

Several studies on human autopsy samples were able to show an effect of the PMI on the mRNA quantity [11, 46] yet other studies have refuted this prediction [36, 47]. In the present study, we found that the groups differing in PMI show significant differences in transcript levels of several genes in cardiac muscle and skeletal muscle tissue (Fig. 2a). Surprisingly, these differences cannot always be explained by a decrease in the relative mRNA quantity in relation to the time period since death. On the contrary, GAPDH in skeletal muscle tissue and B2M and HPRT1 in cardiac muscle tissue show increased relative transcript amounts in samples with higher PMI. On the other hand, in case of GAPDH and HPRT1, this is only true for short post mortem intervals, while in longer periods since death, the expected decrease in relative mRNA amounts becomes clearly visible. In cardiac muscle tissue, PMI was the only parameter showing significant influence on transcript amounts.

Because it has already been shown that the cause of death may influence gene expression patterns [24], we assorted samples according to their donor's cause of death. Beside the PMI, this parameter seems to have the strongest influence on transcript quantities (Fig. 2b). In brain samples, B2M, HPRT1 and TBP show significantly lower mRNA quantities in individuals that died of cardiac death compared to some of the other groups. Also, the cause of death was found to be the only parameter influencing transcript amounts in brain tissue. Whether

or not one of the genes mentioned is a possible candidate for becoming a biomarker for cardiac diseases has to be analysed in a separate study comprising a larger sample set.

In a former study, Preece et al. [47] already pointed out that post mortem mRNA quality in brain tissue varies between males and females, which suggested a possible difference in gene expression between genders. Contrary to these considerations, our study showed only one significant gender-related difference in UBC of post mortem skeletal muscle tissue (Fig. 2c).

In addition to gender, age at death was mentioned as a possible parameter, which may have an impact on post mortem mRNA expression level [47]. Following results from former studies, our analysis showed varying degrees of mRNA level for 18S rRNA and GAPDH in skeletal muscle tissue (Fig. 2d). Concordantly to gender-relating results, our study indicates no further conspicuous difference in gene expression level between younger and older individuals.

After considering obesity as a polygenetic disease, the correlation between individual body mass index and gene expression gains interest in current research [48, 49]. In our sample set, we found significant changes of transcript quantities of ACTB and GAPDH in skeletal muscle. An explanation for this finding may be the anatomical location of the M. iliopsoas and the reduced cooling rate of a body with a higher BMI during storage at 4°C, which could be seen to accelerate degradation of total RNA.

Conclusion

The present study identified a set of appropriate endogenous control genes that can be used for quantitative gene expression analyses in post mortem human cardiac muscle, skeletal muscle and brain tissue. Thus, identified sets of stable reference genes may be used for future gene expression studies as long as the sample population investigated shows a similar composition as the one investigated in this study. As yet, commonly used control genes like ACTB or 18S rRNA turned out to be less stable in post mortem material, which makes them inapplicable for normalisation. Furthermore, this study examined the influence of different parameters on the transcript amount of rather instable genes. Regarding this, especially the cause of death and the post mortem interval showed a considerable impact on gene expression levels.

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References

1. Wallace DC, Fan W (2009) The pathophysiology of mitochondrial disease as modeled in the mouse. Genes Dev 23:1714–1736
2. Gondo Y, Fukumura R, Murata T, Makino S (2009) Next-generation gene targeting in the mouse for functional genomics. BMB Rep 42:315–323
3. Maloney B, Ge YW, Alley GM, Lahiri DK (2007) Important differences between human and mouse APOE gene promoters: limitation of mouse APOE model in studying Alzheimer's disease. J Neurochem 103:1237–1257
4. Bell JE, Alafuzoff I, Al-Sarraj S et al (2008) Management of a twenty-first century brain bank: experience in the BrainNet Europe consortium. Acta Neuropathol 115:497–507
5. Millar T, Walker R, Arango JC et al (2007) Tissue and organ donation for research in forensic pathology: the MRC Sudden Death Brain and Tissue Bank. J Pathol 213:369–375
6. Sandusky G, Dumaual C, Cheng L (2009) Human tissues for discovery biomarker pharmaceutical research: the experience of the Indiana University Simon Cancer Center–Lilly Research Labs Tissue/Fluid BioBank. Vet Pathol 46:2–9
7. Kort EJ, Campbell B, Resau JH (2003) A human tissue and data resource: an overview of opportunities, challenges, and development of a provider/researcher partnership model. Comput Methods Programs Biomed 70:137–150
8. Ravid R (2008) Standard operating procedures, ethical and legal regulations in BTB (Brain/Tissue/Bio) banking: what is still missing? Cell Tissue Bank 9:151–167
9. Heinrich M, Matt K, Lutz-Bonengel S, Schmidt U (2007) Successful RNA extraction from various human post mortem tissues. Int J Legal Med 121:136–140
10. Inoue H, Kimura A, Tuji T (2002) Degradation profile of mRNA in a dead rat body: basic semi-quantification study. Forensic Sci Int 130:127–132
11. Fitzpatrick R, Casey OM, Morris D, Smith T, Powell R, Sreenan JM (2002) Postmortem stability of RNA isolated from bovine reproductive tissues. Biochim Biophys Acta 1574:10–14
12. Catts VS, Catts SV, Fernandez HR, Taylor JM, Coulson EJ, Lutze-Mann LH (2005) A microarray study of post mortem mRNA degradation in mouse brain tissue. Brain Res Mol Brain Res 138 (2):164–177
13. Butte AJ, Dzau VJ, Glueck SB (2001) Further defining housekeeping or maintenance genes focus on a compendium of gene expression in normal human tissues. Physiol Genom 7:95–96
14. Hanson EK, Lubenow H, Ballantyne J (2009) Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. Anal Biochem 387:303–314
15. Haas C, Klesser B, Maake C, Bär W, Kratzer A (2009) mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR. Forensic Sci Int Genet 3:80–88
16. Setzer M, Juusola J, Ballantyne J (2008) Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains. J Forensic Sci 53:296–305
17. Juusola J, Ballantyne J (2007) mRNA profiling for body fluid identification by multiplex quantitative RT-PCR. J Forensic Sci 52:1252–1262
18. Zubakov D, Hanekamp E, Kokshoorn M, van Ijcken W, Kayser M (2008) Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples. Int J Legal Med 122:135–142
19. Bauer M, Patzelt D (2008) Identification of menstrual blood by real time RT-PCR: technical improvements and the practical value of negative test results. Forensic Sci Int 174:55–59
20. Ferrer I, Martinez A, Boluda S, Parchi P, Barrachina M (2008) Brain banks: benefits, limitations and cautions concerning the use

of post mortem brain tissue for molecular studies. Cell Tissue Bank 9:181–194
21. 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 9:46–56
22. Parlapani E, Schmitt A, Wirths O, Bauer M, Sommer C, Rueb U, Skowronek MH, Treutlein J, Petroianu GA, Rietschel M, Falkai P (2008) Gene expression of neuregulin-1 isoforms in different brain regions of elderly schizophrenia patients. World J Biol Psychiatry 7:1–8
23. Kochanowska I, Chaberek S, Wojtowicz A, Marczynski B, Wlodarski K, Dytko M, Ostrowiski K (2007) Expression of genes for bone morphogenetic proteins BMP-2, BMP-4 and BMP-6 in various parts of the human skeleton. BMC Musculoskelet Disord 8:128–127
24. Ikematsu K, Tsuda R, Nakasono I (2006) Gene response of mouse skin to pressure injury in the neck region. Leg Med (Tokyo) 8:128–131
25. Bustin SA, Benes V, Garson JA et al (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622
26. Huggett J, Dheda K, Bustin S, Zumla A (2005) Real time RT-PCR normalisation; strategies and considerations. Genes Immun 6:279–284
27. Hoogewijs D, Houthoofd K, Matthijssens F, Vandesompele J, Vanfleteren JR (2008) Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in C. elegans. BMC Mol Biol 9:9–16
28. Heinrich M, Lutz-Bonengel S, Matt K, Schmidt U (2007) Real-time PCR detection of five different "endogenous control gene" transcripts in forensic autopsy material. FSI: Genetics 1:162–169
29. 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):research0034.1–research0034.11
30. 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 1123:1–11
31. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 7:3–16
32. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:E45
33. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 8:R19
34. Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169–193
35. Tanic N, Perovic M, Mladenovic A, Ruzdijic S, Kanazir S (2007) Effects of aging, dietary restriction and glucocorticoid treatment on housekeeping gene expression in rat cortex and hippocampus-evaluation by real time RT-PCR. J Mol Neurosci 32:38–46
36. Gutala RV, Reddy PH (2004) The use of real time PCR analysis in a gene expression study of Alzheimer´s disease post mortem brains. J Neuroscie Methods 132:101–107
37. Lupberger J, Kreuzer KA, Baskaynak G, Peters UR, le Coutre P, Schmidt CA (2002) Quantitative analysis of beta-actin, beta-2-microglobulin and porphobilinogen deaminase mRNA and their comparison as control transcripts for RT-PCR. Mol Cell Probes 16:25–30
38. Hamalainen HK, Tubman JC, Vikman S et al (2001) Identification and validation of endogeneous reference genes for expression profiling of T helper cell differentiation by quantitative real time RT-PCR. Anal Biochem 299:63–70
39. Tsuji N, Kamagata C, Furuya M et al (2002) Selection of an internal control gene for quantification of mRNA in colonic tissues. Anticancer Res 22(6c):4173–4178
40. Bas A, Forsberg G, Hammarström S, Hammarström ML (2004) Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. Scand J Immunol 59:566–573
41. Schmid H, Cohen CD, Henger A, Irrang S, Schlömdorff D, Kretzler M (2003) Validation of endogeneous controls for gene expression analysis in microdissected human renal biopsies. Kidney Int 64:356–360
42. Gorzelniak K, Janke J, Engeli S, Sharma AM (2001) Validation of endogeneous controls for gene expression studies in human adipocytes and preadipocytes. Horm Metab Res 33:625–627
43. Hederstedt L, Rutberg L (1981) Succinate dehydrogenase—a comparative review. Microbiol Rev 45:542–555
44. Suzuki T, Higgins PJ, Crawford DR (2000) Control selection for RNA quantitation. Biotechniques 29:332–337
45. Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 313:856–862
46. Zhai QH, Ruebel K, Thomson GB, Lloyd RV (2003) Androgen receptor expression in C-cells and in medullary thyroid carcinoma. Endocr Pathol 14:159–165
47. Preece P, Cairns NJ (2003) Quantifying mRNA in post mortem human brain: influence of gender, age at death, post mortem interval, brain pH, agonal state and inter-lobe mRNA variance. Brain Res Mol Brain Res 118:60–71
48. Aberele J, Flitsch J, Beck NA, Mann O, Busch P, Peitsmeier P, Beil FU (2008) Genetic variation may influence obesity only under conditions of diet: analysis of three candidate genes. Mol Genet Metab 95:188–191
49. Perez-Matute P, Neville MJ, Tan GD, Frayn KN, Karpe F (2009) Transcriptional control of human adipose tissue blood flow. Obesity (Silver Spring) 17:681–688