Normalization Strategy for Selection of Reference Genes for RT-qPCR Analysis in Left Ventricles of Failing Human Hearts

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Research Article

Keywords: failing hearts, left ventricles, qPCR, reference gene, normalization

Posted Date: September 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-850536/v1>

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Version of Record: A version of this preprint was published at BMC Cardiovascular Disorders on April 19th, 2022. See the published version at <https://doi.org/10.1186/s12872-022-02614-9>.

Abstract

Introduction: Quantitative RT-PCR is a valuable tool for assessing the gene expression in different human tissues, particularly due to its exceptional sensitivity, accuracy and reliability. However, the choice of adequate control for normalization is a crucial step, greatly affecting the results of all subsequent analyses. So far, only a few studies were focused on the selection of optimal reference genes in left ventricles of failing human hearts, leading to several disparities in experimental results focused on differential gene expression in this area. Therefore, the main objective of this study was to identify a set of suitable reference genes in normal and failing left ventricle tissues, which could increase the reliability of RT-qPCR-based studies in the future.

Methods: We analyzed the expression of 15 commonly used housekeeping genes (ACTB, B2M, GAPDH, GUSB, HMBS, HPRT1, IPO8, PGK1, POLR2A, PPIA, RPLP0, TBP, TFRC, UBC and YWHAZ) in left ventricles of normal and failed hearts with two-step approach. In the first step, we excluded genes which are variably expressed using ANOVA-based statistical method. Afterwards, the remaining genes were analyzed using geNorm, NormFinder and BestKeeper algorithms, together with comparative delta Cq method. Finally, the geometric mean of gene rankings across all five methods was calculated.

Results: Our analysis identified IPO8, PGK1 and POLR2A as the most stably expressed genes, whereas ACB2 and B2M were found to be expressed variably, suggesting a potential role of these genes in the pathophysiological processes in failing human hearts.

Discussion/Conclusion: Using our two-step approach, we identified and validated three reference genes expressed invariantly in left ventricles of both healthy and failing human hearts, as well as provided a guideline for the selection of reference genes in studies comparing gene expression in these types of tissues.

Introduction

Heart failure (HF) is a serious clinical disorder and the most common reason for hospitalization and death among older adults in Europe and the United States [1,2]. In numerous types of heart diseases, including ischemic heart disease, dilated cardiomyopathy and coronary artery disease, ventricular remodeling is a major feature of heart failure [3,4]. As the remodeling progresses, heart changes its shape and morphology, mainly due to pathological processes such as cardiomyocyte hypertrophy, apoptosis, inflammation and collagen deposition [5,6]. Although there is a significant progress in understanding of the complex pathophysiology of HF, the biochemical pathways associated with the cardiac remodeling haven't been fully described yet.

Over the last fifteen years, the differences in gene expression between normal and failing human hearts were thoroughly studied, leading to the identification of many genes potentially involved in the ventricular remodeling [6,7]. In most such studies, the quantification of mRNA abundance in different heart compartments plays a crucial role and quantitative reverse transcriptase polymerase chain reaction (RT-

qPCR) is widely considered the method of choice because of its high sensitivity and reliability [8]. However, this method needs an accurate normalization to differentiate between the technical variations and the true biological differences [9]. Generally, this is achieved by relating the mRNA levels of studied genes to those of internal reference genes (RGs), whose expression is unaffected by the biological event under investigation. Previous studies have shown, that the expression levels of frequently used reference genes, such as GAPDH or 18S rRNA can vary markedly across species, cells or tissues, and are often modulated by the pharmacological treatment and/or experimental conditions [10,11]. Therefore, using just one gene for normalization could lead to disparities in results and cause problems in downstream analyses. To avoid this, two or more RGs are commonly used for accurate quantification of gene expression [12].

In order to identify the best-suited reference genes for normalization, several statistical algorithms such as geNorm, NormFinder or BestKeeper were developed and are widely accepted [12-14]. Based on the core properties of these algorithms, they could be divided into (i) pairwise comparison-based (geNorm, BestKeeper), which compare the expression of genes in pairs regardless of whether the analyzed sample belongs to the control or tested group, and (ii) the variance-based (NormFinder), which statistically evaluate the intragroup and intergroup variation in gene expression. Nowadays, geNorm and NormFinder are most commonly used and both of them produce reliable results. However, a key assumption that none of the candidate reference genes shows systematic variation in its expression profile across the tested samples, must be fulfilled [15-17]. Since this assumption is very frequently violated in the practice, the candidate reference genes (CRGs) should be tested to confirm that their expression do not vary under the specific experimental conditions [15].

Over the past decade, only a few reports on the identification of stably expressed genes in left ventricles (LV) of failing and control human hearts have been published [18-20]. This is partially due to the restrictive numbers of donors, especially when the healthy heart tissue is concerned. These studies led to the identification of various combinations of RGs used for normalization, depending on differences in the number of samples collected, pathophysiological background of the samples, pharmacological treatment, technology, and normalization strategy. Moreover, the number of CRGs analyzed, together with their metabolic characteristics also vary in different reports [10,18-23]. Therefore, we believe that the analysis of a larger palette of candidate reference genes in HF samples of LV tissue will provide an important overview of CRGs which should be considered for normalization when analyzing the gene expression by RT-qPCR in LV of failing hearts.

The aim of this study was to identify the suitable genes in HF patient samples with different pathological background (ischemic heart disease, hypertrophic cardiomyopathy, dilated cardiomyopathy and coronary artery disease) and compare the results with those already published. We analyzed 15 genes commonly used for normalization (ACTB, B2M, GAPDH, GUSB, HMBS, HPRT1, IPO8, PGK1, POLR2A, PPIA, RPLP0, TBP, TFRC, UBC and YWHAZ) and applied a step-by-step approach, where the CRGs were first tested for their expression variation between the groups with ANOVA and then analyzed by four different algorithms to identify the most stably expressed ones. We also show that the 3 best scoring candidates can be used

as reference genes for studying the differential gene expression of MYH6 and MYH7 and discuss the potential role of B2M and ACTB in the pathological processes in LV of failing human hearts.

Materials And Methods

Biological samples and clinical characteristics

Tissue samples were taken from left ventricular myocardium from non-failing and failing human hearts. Failing hearts were obtained from patients undergoing heart transplantation for end-stage heart failure, resulting from ischemic heart disease (n=5, all male), hypertrophic cardiomyopathy (n=4, 2 female and 2 male), dilated cardiomyopathy (n=10, all male) and coronary artery disease (n=9, all male). The clinical characteristics of patients prior to the heart transplantation are summarized in Table 1. Healthy heart tissue was obtained from unmatched donors, (n=5, 3 female, 2 male) suffering from subarachnoid hemorrhage and head injury. No donor had previous diagnosed heart pathology. The average age of healthy donors was 29 year, (22 – 44 years). All samples were immediately frozen in liquid nitrogen and store at -80°C until the RNA isolation.

Candidate reference genes

For this study, the candidate reference genes were selected based on previous RT-qPCR studies [18]. Specifically, ACTB, B2M, GAPDH, GUSB, HMBS, HPRT1, IPO8, PGK1, POLR2, PPIA, RPLP0, TBP, TRFC, UBC, and YWHAZ were analyzed. Detailed information on genes and technology used are presented in Supplementary Table 1.

RNA preparation and cDNA synthesis

Total RNA from frozen samples was isolated using TriReagent (ThermoFisher Scientific, USA) according to the manufacturer's instructions. The RNA concentration and purity were evaluated spectrophotometrically using Nanodrop ND-100UV-Vis Spectrophotometer (ThermoFisher Scientific, USA). All samples used in subsequent enzymatic reactions showed a 260/280 nm absorbance ratio of 1.8 – 2.2. The integrity of the RNA was assessed on Qubit 4 Fluorimeter using Qubit RNA IQ Assay (ThermoFisher Scientific, USA). First strand cDNA was synthesized with High Capacity Reverse Transcription Kit (ThermoFisher Scientific, USA) according to the manufacturer's manual. For each sample, 200 ng of total RNA was reverse transcribed into cDNA. Reverse transcription reactions were diluted to 1 ng/μl and stored until use at -80°C.

Real-Time PCR

Real Time PCR reactions were performed using TaqMan Gene Expression Assays (Supplementary Table 1) and TaqMan Universal Master Mix II (both ThermoFisher Scientific, USA). Each reaction contains 1 ng of cDNA, 0.5x TaqMan Gene expression Assay, and 1x TaqMan Universal Master Mix II in 20 μl total volume. Reactions were amplified on QuantStudio™ 5 Real-Time PCR System (ThermoFisher Scientific,

USA) in triplicate for each sample and PCR cycling conditions were used according to manufacturer instructions. The amplification efficiency was assessed using LinRegPCR software [24].

Reference gene stability evaluation

The expression of 15 selected CRGs was tested as proposed in [15]. For each CRG, the C_q values were converted to relative expression ratios (Rs), followed by transformation to their natural logs and subjected to one-way ANOVA. The mean squares of groups (MSB) which represent the intergroup variance caused by differences between groups (healthy and failing) and mean square errors (MSW), which represent the intragroup variance, were calculated. Consequently, the MSB and MSW of each CRG were divided by $-1/\bar{X}$ to calculate the intergroup variation index (VB) and intragroup variation index (VW) for each CRG ($\bar{X}$ is the average of natural logarithmic transformed relative expression ratios of the corresponding CRG). A stability index was subsequently computed by multiplying VB by VW. Afterwards, all CRGs with F-significance levels below 0.05 were excluded from further analyses, while the remaining CRGs were analyzed employing four different approaches:

- 1) We ranked the CRGs by their M-value (average pair-wise variation of specific gene with all of the other analyzed genes) calculated using geNorm algorithm (implemented as a module in Qbase software).
- 2) We used NormFinder algorithm (included as add-in excel sheet) to calculate the stability values of CRGs and rank them accordingly (the lowest stability value represents the most stably expressed gene).
- 3) The BestKeeper software was used to calculate Pearson coefficient and p-value for each CRG (for each gene, p-value was <0.05). Subsequently, Pearson coefficients were correlated with BestKeeper index and the resulting correlation coefficients were used as the basis for the ranking (the highest correlation coefficient was scored as no. 1 in the ranking list).
- 4) Delta C_q method was used to calculate mean ΔC_q and SD across all pairwise CRG comparisons. Subsequently, mean SD was calculated across all comparisons of the gene to all of the other remaining CRGs and the gene with the lowest mean SD was considered the most stable one.

The final ranking of CRGs was then calculated as a geometric mean of ranking of each individual CRG in all particular rankings and the top three CRGs were considered the most stable genes in our dataset [18].

Statistical analysis

Stability indexes in ANOVA frame were calculated using XLSTAT software [25] on amplification corrected and log transformed data as proposed in [15]. Raw amplification data (C_q values) from qPCR were exported into Qbase software [26], normalized, log transformed and the Student t-test was performed to evaluate statistical differences in two independent groups using Qbase statistical module. The power of the test was assessed by XLSTAT software. The p-values ≤ 0.05 were considered as significant. The numerical data were visualized with GraphPad software [27] as mean $\pm$ S.E.M.

Results

The expression ranges of all CRGs in 33 samples from left ventricles of human hearts (5 healthy, 28 diseased) are shown in Supplementary Table 2. All selected genes are expressed in human myocardium and their respective Ct values vary significantly with B2M and GAPDH exhibiting the highest expression levels and GUSB showing the lowest expression. The smallest Ct range was associated with HPRT1 gene, whereas ACTB showed the largest. The stability of CRGs was first analyzed using ANOVA-based approach (Table 2), showing a variability of stability indexes, ranging from 0.006 (ACTB) to 0.232 (IPO8). Importantly, this analysis also revealed a significant difference in gene expression of B2M, YWHAZ, TBP, RPLP0 and ACTB between the tested groups, questioning their reliability as RGs. Based on these data, the five genes were excluded from further analyses.

Remaining 10 CRGs were analyzed with geNorm, NormFinder and BestKeeper algorithms, as well as the Delta Cq method. When comparing the two pairwise comparison-based systems (geNorm and BestKeeper), it is clear that both of them scored IPO8 and POLR2A among the most stably expressed genes (Fig. 1, Supplementary Table 3). Moreover, when analyzed with geNorm, all selected CRGs showed M-values below the threshold of 0.5, indicating high stability (Supplementary Table 3). On the other hand, the ranking of several genes such as GAPDH or HMBS remains questionable, since their position varies between the two ranking lists. Ranking pattern similar to those generated by geNorm and BestKeeper was also observed using Delta Cq method, identifying the same five most stably expressed CRGs as in case of geNorm. Although the specific ranking position of the individual genes varies slightly between the two approaches, IPO8 and POLR2A were again present among the top three candidates (Fig.1). As a complementary approach, we employed the variance-based NormFinder algorithm, which accounts for variation in gene expression among and within the sample groups and avoids the misinterpretation of data caused by artificial selection of co-regulated genes [13]. Interestingly, the comparison of stability values generated by NormFinder suggests the combination of GUSB and UBC as the optimal set of RGs, since they showed the lowest combined stability value. Individually, IPO8, PGK1 and HPRT1 were listed as the three most stably expressed CRGs (Fig. 1, Supplementary Table 3).

Finally, taking to account the position in every scoring list, we calculated the final ranking for each tested CRG (using the geometric mean). As expected, IPO8 was identified as the most stably expressed gene in the entire dataset (each method except for geNorm identified IPO8 as the most stably expressed gene). The positions of other tested CRGs were much more variable, although multiple algorithms proposed POLR2A (geNorm, BestKeeper, Delta Cq) and PGK1 (ANOVA, NormFinder) as the other two CRGs with high stability of expression (Table 3). Taken together, our combined approach, employing a variety of algorithms to compare the stability of tested CRGs showed differences in expression levels of some CRGs between healthy and diseased human hearts and propose IPO8, PGK1 and POLR2A as the optimal RGs for RT-qPCR studies of mRNA levels in failing human hearts.

Analysis of MYH6 and MYH7 genes expression using top three CRGs

To validate the 3 top-scoring CRGs, we tested our normalization strategy in RT-qPCR analysis of MYH6 and MYH7 genes expression in LV of failing human hearts. MYH6 and MYH7 genes encode the two myosin heavy chain isoforms (α -MHC and β -MHC, respectively), whose expression is altered in diseased tissues and therefore represent a suitable control for our experimental strategy. Specifically, MYH6 gene is significantly downregulated in failing heart tissue, while MYH7 is upregulated [28-30]. As expected, the results presented in Figure 2 and Supplementary Table 4 showed that the transcription of MYH6 gene is downregulated on average 7x, while MYH7 is upregulated 4.5x, which is in agreement with previously published data [28-30] and further support IPO8, PGK1 and POLR2A as reliable RGs. The power of the test reached 100% for both genes.

Analysis of ACTB and B2M genes expression using top three CRGs

The analyses of CRGs in several previous reports showed that ACTB and B2M genes, often used as RGs, are among the least stably expressed CRGs in human left ventricles, regardless of clinical or experimental conditions used [18,20-21]. This is in concert with our data, which showed significant differences in expression of these genes in LV of healthy and failed heart tissue (Table 2). Furthermore, we compared the levels of mRNA of both genes by RT-qPCR, using IPO8, PGK1 and POLR2A as RGs (Fig. 3, Supplementary Table 5), revealing a significant down-regulation of both genes at the transcription level in left ventricles of failing hearts (the power of the test for both genes reached 100%). This observation suggest that B2M and ACTB are not suitable as RGs in gene expression studies aimed at LV of failing human hearts and the changes in their expression might be connected to pathological processes leading to heart failure.

Discussion

The RT-qPCR is a powerful tool to study gene expression at mRNA level. As many factors can influence the accuracy of results obtained by this method (RNA quality, efficiency of reverse transcription, primer design), a correct normalization of primary data is necessary to reduce the variations caused by experimental and analytical procedures. Accordingly, the selection of proper reference genes is crucial, as these genes directly influence the interpretation of RT-qPCR results. Furthermore, in clinical HF samples, the variations in gene expression can be also affected by the number of samples analyzed, specific pathophysiology of the samples and pharmacological treatment, as well as the normalization strategy. Therefore, it is not surprising that the final choice of RGs selected for normalization in various studies of LV from normal and failing heart tissues differ significantly [10,18-23,31,32].

In this study, we selected 15 most commonly used CRGs and compared their stability in both the healthy and diseased samples. First, we employed an ANOVA-based approach [15] and F-statistics to calculate the stability index for each CRG (Table 2). Based on these calculations, IPO8, PGK1 and HPRT1 were considered the most stable genes, with nonsignificant p-values and the lowest intergroup variance. This analysis also revealed that 5 genes (TBP, YWHAZ, ACTB, B2M, RPLP0) were expressed variably ($p \leq 0.05$) and thus were excluded from further analyses. Among these genes, TBP was previously reported as

stably expressed only in post mortem human cardiac muscle tissue [21], while in other studies where it was analyzed, it was never ranked among the most stably expressed genes [18,31]. On the other hand, YWHAZ was a top-ranked RG when samples from left and right ventricles of patients with LVAD (left ventricular assist device) or HT (heart transplantation without previously implanted LVAD) were tested [22]. Moreover, along with GAPDH, IPO8, POLR2A and PPIA, YWHAZ was identified as a suitable RG in several heart cavities and disease conditions [18]. In contrast, the identification of endogenous controls in LV of hearts from organ donors ranked YWHAZ among the less stable genes [31], which is in concert with our analysis and questions its role as RG in studies aimed at the differential gene expression in LV of failing hearts. The RPLP0 was described as suitable RG in study of hearts from human donors [32], but not in the study concerning normal and diseased hearts [18]. Regarding these genes, however, we would like to note that no data have been reported as to whether they had been tested for systematic variation or not. Therefore, we cannot exclude the possibility that in other clinical studies on LV of failing human hearts including different sets of cardiomyopathies and/or different sizes of both control and diseased groups, no statistical significant difference between the tested groups will be observed. Hence, we propose to include these genes in the CRG sets used for the identification of the most stably expressed genes in LV of normal and failing hearts in the future. Importantly, this does not apply to the last two genes, ACTB and B2M (see below).

Afterwards, the 10 stably expressed CRGs were further analyzed with geNorm, NormFinder and BestKeeper algorithms, as well as the Delta Cq method. Since geNorm, NormFinder or a combination of both is used in the majority of studies searching for suitable RGs, we used them as the primary tools and compared the resulting ranking lists with those generated by BestKeeper and Delta Cq (Fig. 1). Both of these algorithms provide reliable results if none of the evaluated genes shows a systematic variation in the expression profile during experiment. Unfortunately, there is no way to predict whether a particular CRG is expressed invariantly before performing the experiment and the preliminary analyses of CRGs are frequently based on publicly unavailable microarray or RNAseq data. One way to overcome this problem is to use the statistical approach, which evaluates each CRG individually and identifies genes with systematic variation in gene expression. Using all 5 aforementioned methods, we generated individual ranking lists for each algorithm and eventually combined them into the final ranking. This analysis identified IPO8, PGK1 and POLR2A as the most stably expressed genes, suggesting they might serve as reliable RGs in RT-qPCR analyses (Table 3).

Among the other seven genes (ranked 4.-10.), HPRT1 has been previously selected for normalization of gene expression profiles in cardiac tissue and blood cells in the study of myocarditis [10,19], PPIA was shown to be a functional internal control in study of healthy organ donors [31] and cytokine expression in patients with LVAD [22]. Interestingly, the results on GAPDH are contradictory, as it was reported as reliable RG in three separate studies [19, 20, 31], while in one report it was found to be the least stable gene [32]. In our study, GAPDH was scored as a less stable gene, ranked sixth in the final calculation. This is in agreement with results of [18], where GAPDH was ranked as a less stable gene in LV of control and failing hearts. On the other hand, the same study showed that GAPDH is a stable reference gene across different heart cavities and disease conditions [18]. GUSB has been analyzed only in one study, where it

was described as the second most stable gene in LV of human controls and failing heart tissues [18]. This is inconsistent with the results of our analysis, where GUSB was ranked among the less stable genes. The UBC gene was analyzed in three studies [18,21,31], always found as a less stable, whereas the HMBS gene was reported as a suitable RG in postmortem human cardiac muscle tissue, but not in sudden cardiac death (SDC) analysis [21,23]. Together with TFRC gene, HMBS was found as the least stable CRG in our analysis, occupying the last two positions of the final ranking.

Finally, when comparing the top three RGs from our study (IPO8, PGK1 and POLR2A) with previously published reports, IPO8 and POLR2A were described as suitable RGs in left ventricles of failing hearts and also in all heart chambers and disease conditions [18]. PGK1 has been also reported as appropriate internal control in two analyses of human heart failure [10,18], suggesting all of these genes might serve as reliable RGs.

Analysis of MYH6 and MYH7 genes expression using top three CRGs

To experimentally test these three RGs, we analyzed the expression of MYH6 (downregulated in LV of failing hearts) and MYH7 (upregulated in LV of failing hearts) genes by RT-qPCR. Even though the control samples showed a slight variation in expression of MYH6, the expression of both MYH6 and MYH7 was significantly altered in LV of failing hearts, showing the expected patterns of differential regulation (Fig. 2). These data indicate that selected RGs are appropriate for examining the differential gene expression in proposed experimental set up and might be used in RT-qPCR analyses aimed at the identification of both upregulated and downregulated genes of interest.

Analysis of ACTB and B2M genes expression using top three CRGs

ACTB and B2M genes are very frequently used as RGs in gene expression studies. In our analysis, however, both showed a highly variant expression and were excluded from the dataset in the first step due to low p-values and high ANOVA stability indexes (Table 2). Similarly, these genes were also found as less stably expressed in other studies focusing on the identification of RGs in LV of failing human hearts, regardless of their clinical background [10,18,20-21,31]. Interestingly, the significant decrease in expression of these two genes might be directly associated with the pathophysiology of the LV of failing heart tissue.

In case of B2M gene, it has been recently shown that high levels of B2M protein in serum are associated with severity of coronary artery disease without renal dysfunction [33]. In addition, B2M levels are higher in plasma of patients hospitalized with chronic heart failure and correlate with the severity of cardiorenal failure [34]. Recently, the increased expression of B2M in stressed mouse cardiomyocytes was reported to stimulate a wound healing response in mouse fibroblasts with induced ischemia-reperfusion injury [35]. In contrast to these observations, we found the B2M mRNA levels decreased in failed hearts. One

possible explanation of this discrepancy is that decreased expression of B2M gene could lead to a decreasing ability of wound healing in end-stage human hearts. However, the exact role of B2M gene in pathophysiology of human hearts remain to be investigated.

The role of ACTB gene in failing human hearts is also not yet fully understood. ACTB is one of the cytoskeletal proteins and participates in many important cellular processes, including muscle contraction, cell motility and division, organelle movement, the establishment and maintenance of cell junctions and cell shape [36]. The disorganization of cytoskeletal proteins and loss of sarcomeric structures contribute to the pathogenesis of contractile dysfunction in mammalian hearts and the decreased expression of this gene might lead to the loss of contractile force in end-stage hearts and subsequent heart failure [37]. Moreover, in many eukaryotic cells, β -actin (protein product of ACTB gene) is also present in nucleus, where it regulates the expression of specific genes and processes connected to cell division, and proliferation [38,39]. One of the best-characterized examples of β -actin role in the regulation of nuclear complexes is its involvement in regulation of MRTF (myocardin-related transcription factor) system, an activator of SRF (serum response factor). SRF is a regulator of cardiac gene expression in response to hypertrophic stimuli and several processes which reduces the monomer-actin (G-actin) lead to the retention of MRTF in nucleus and SRF activation. This in turn could lead to the expression of genes responsible for cardiac remodeling [40,41]. Interestingly, the cardiac remodeling might be also regulated via interaction of β -actin with endothelial nitric oxide synthase (eNOS). The association of eNOS with β -actin increases eNOS activity and the nitric oxide (NO) production [41]. The following activation of cGMP-dependent protein kinase (PKG) by nitric oxide (NO) might inhibit the hypertrophic signaling pathways and act as pro-hypertrophic brake [5,42].

Conclusion

In this study, we identified the most stably expressed reference genes for normalization of transcription profiles in LV of healthy and failing human hearts of different pathological background. We analyzed 15 commonly used housekeeping genes, which should exhibit a minimal differential expression across various tissues. In the first step, we eliminated variably expressed CRGs using ANOVA-based statistical approach and analyzed the remaining genes with four different algorithms. Afterwards, we combined the ranking positions of individual CRGs from all methods to create the final list. Using this approach, we identified IPO8, PGK1 and POLR2A as the most stably expressed CRGs in our sample set, suggesting them as reliable RGs for transcription profiling in LV of failing and healthy hearts. We also found that ACTB and B2M are variably expressed in our samples, which is in agreement with several studies focused on selection of proper normalization strategy in human myocardium. Since this may be directly related to their association with cardiac remodeling and pathology development in failing hearts, we do not recommend these genes as RGs in the future studies. We also conclude, that our two-step approach based on testing the invariant expression of each CRG and the subsequent analysis with several normalization algorithms is a suitable approach to identify reliable and stable reference genes in left ventricles of healthy and failing human hearts.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki principles and approved by the Ethical Committees of The National Institute for Cardiovascular Diseases in Bratislava, Slovakia (No. IK-NP-0021-24/1426/14 and NUSCH EK 126/180509). After learning about the purpose and nature of the experiments, an informed written consent was obtained from all patients before the cardiac surgery. Afterwards, the personal data were handled in accordance with GDPR directive and the legal system of Slovak Republic (Personal Data Protection Act, Act No. 18/2018 Collections of Laws).

Consent for publication

Not applicable.

Availability of data and material

All data analyzed in this study are included in the article and/or its supplementary materials. Further enquiries can be directed to the corresponding author.

Competing interests

The authors have no competing interests to declare.

Funding

This study was supported by APVV-00-14-416 grant of Slovak Research and Development Agency.

Author Contributions

Z. Červenák, B. Šalingová and A. Adamičková performed the experiments, A. Gažová collected the samples and has conducted the statistical analysis. Z. Červenák and F. Červenák wrote the manuscript. J. Kyselovič supervised the project.

Acknowledgement

We thank to Dr. Gabriel Dóka for his comments on the experimental design and statistical analysis.

References

1. Dickstein K, Cohen-Solal A, Filippatos G, McMurray JJV, Ponikowski P, Poole-Wilson PA, et al. ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008. *Eur Heart J*. 2008 Mar;29(19): 2388-2442. <https://doi.org/10.1093/eurheartj/ehn309>
2. Jenck SF, Williams MW, Coleman EA. Rehospitalizations among patients in the Medicare fee-for-service program. *N Engl J Med*. 2009 Apr;360(14):1418-28. <https://doi.org/10.1056/NEJMsa0803563>

3. Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling-concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling. *J Am Coll Cardiol*. 2000 Mar;35(3):569-82. [https://doi.org/10.1016/s0735-1097\(99\)00630-0](https://doi.org/10.1016/s0735-1097(99)00630-0)
4. Shah AM, Mann DJ. In search of new therapeutic targets and strategies for heart failure: recent advances in basic science. *Lancet*. 2011 Aug;378(9792):704-712. [https://doi.org/10.1016/S0140-6736\(11\)60894-5](https://doi.org/10.1016/S0140-6736(11)60894-5)
5. Hill JA, Olson EN. Cardiac plasticity. *N Engl J Med*. 2008 Mar;358(13):1370-1380. <https://doi.org/10.1056/NEJMra072139>
6. Dirkx E, da Costa Martins PA, De Windt LJ. Regulation of fetal gene expression in heart failure. *Biochim Biophys Acta* 2013 Dec;1832(12):2414-2424. <https://doi.org/10.1016/j.bbadis.2013.07.023>
7. Szibor M, Pöling J, Warnecke H, Kubin T, Braun T. Remodeling and dedifferentiation of adult cardiomyocytes during disease and regeneration. *Cell Mol Life Sci*. 2014 May;71(10):1907-16. <https://doi.org/10.1007/s00018-013-1535-6>
8. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. *Nat Protoc*. 2006;1(3):1559-1582. <https://doi.org/10.1038/nprot.2006.236>
9. Derveaux S, Vandesompele J, Hellemans J. How to do successful gene expression analysis using real-time PCR. *Methods* 2010 Apr;50(4):227-230. <http://doi.org/10.1016/j.ymeth.2009.11.001>
10. Brattelid T, Winner LH, Levy FO, Liestol K, Serjested OM, Andersson KB. Reference gene alternatives to GAPDH in rodent and human heart failure gene expression studies. *BMC Mol Biol*. 2010 Mar;11:22. <https://doi.org/10.1186/1471-2199-11-22>
11. Everaert BR, Boulet GA, Timmermans JP, Vrints CJ. Importance of Suitable Reference Gene Selection for quantitative real-time PCR: Special reference to mouse myocardial infarction studies. *PLoS ONE* 2011;6(8):e23793. <https://doi.org/10.1371/journal.pone.0023793>
12. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002 Jun;3(7):RESEARCH0034. <https://doi.org/10.1186/gb-2002-3-7-research0034>
13. Andersen CL, Jensen JL, Orntoft TF. 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*. 2004 Aug;64(15):5245–5250. <https://doi.org/10.1158/0008-5472.CAN-04-0496>
14. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pairwise correlations. *Biotechnol Lett*. 2004 Mar;26(6):509–515. <https://doi.org/10.1023/b:bile.0000019559.84305.47>

15. Khanlou KM, Bockstaele EV. A critique of widely used normalization software tools and an alternative method to identify reliable reference genes in red clover (*Trifolium pratense* L.). *Planta*. 2012 Nov; 236(5):1381-1393.
[https://doi.org/ 10.1007/s00425-012-1682-2](https://doi.org/10.1007/s00425-012-1682-2)
16. Kortner TM, Valen EC, Kortner H, Marjara IS, Krogdahl A, Bakke AM. Candidate reference genes for quantitative real-time PCR (qPCR) assays during development of a diet-related enteropathy in Atlantic salmon (*Salmo salar* L.) and the potential pitfalls of uncritical use of normalization software tools. *Aquaculture* 2011;318:355-363. <https://doi.org/10.1016/j.aquaculture.2011.05.038>
17. Sundaram VK, Sampathkumar NK, Massaad C, Grenier J. Optimal use of statistical methods to validate reference gene stability in longitudinal studies. *PLoS ONE* 2019 Jul;14(7):e0219440.
<https://doi.org/10.1371/journal.pone.0219440>
18. Molina CE, Jacquet E, Ponien P, Munoz-Guijosa Ch, Baczkó I, Maier LS, et al. Identification of optimal reference genes for transcriptomic analysis in normal and diseased human heart. *Cardiovasc Res*. 2018 Feb;114(2):247-258. <https://doi.org/10.1093/cvr/cvx182>
19. Runov AL, Kurchakova EV, Khaschevskaya DA, Moiseeva OM, Vonsky, MS. Selection of reference genes for transcription analysis for myocarditis studies. *Tsitologiya*, 2015; 57 (3), 212–217. PMID 26021171
20. Li M, Rao M, Chen K, Zhou J, Song J. Selection of reference genes for gene expression studies in heart failure for left and right ventricles. *Gene* 2017 Jul;620:30-35.
<https://doi.org/10.1016/j.gene.201704.006>
21. Koppelkam A, Vennemann B, Fracasso T, Lutz-Bonengel S, Schmidt U, Heinrich M. Validation of adequate endogenous reference genes for the normalisation of qPCR gene expression data in human post-mortem tissue. *Int J Legal Med*. 2010 Sep;124:371-380. [https://doi.org/ 10.1007/s00414-010-0433-9](https://doi.org/10.1007/s00414-010-0433-9)
22. Caselli C, D`amico A, Caruso R, Cabiati M, Prescimone T, Coyyi L, et al. Impact on normalization strategy on cardiac expression of pro-inflammatory cytokines: Evaluation of reference genes in different human myocardial regions after Left Ventricular Assist Device. *Cytokine* 2013 Aug;63(2):113-122. [https://doi.org/ 10.1016/j.cyto.2013.04.021](https://doi.org/10.1016/j.cyto.2013.04.021)
23. Xue Y, Zhao R, Du SH, Zhao D, Li DR, Xu JT, et al. Decreased mRNA levels of cardiac Cx43 and ZO1 in sudden cardiac death related to coronary atherosclerosis: a pilot study. *Int J Legal Med*. 2016 Jul;130(4):915-922.
[https://doi.org/ 10.1007/s00414-016-1353-0](https://doi.org/10.1007/s00414-016-1353-0)
24. Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, van den Hoff MJB, et al. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res*. 2009 Apr;37(6):e45.
[https://doi.org/ 10.1093/nar/gkp045](https://doi.org/10.1093/nar/gkp045)
25. Addinsoft (2021). XLSTAT statistical and data analysis solution. New York, USA.
<https://www.xlstat.com>

26. Hellemans J, Mortier G, Paepe AD, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 2007;8(2):R19. <https://doi.org/10.1186/gb-2007-8-2-r19>
27. GraphPad Prism version 9.1.1. for Windows, GraphPad Software, San Diego, California USA. www.graphpad.com.
28. Nakao K, Minobe W, Roden R, Bristow MR, Leinwald LA. Myosin heavy chain gene expression in human heart failure. *J. Clin Invest.* 1997 Nov;100(9):2362-2370. <https://doi.org/10.1172/JCI119776>
29. Lowes BD, Minobe W, Abraham WT, Rizeq MN, Bohlmeier TJ, Quaife RA, et al. Changes in gene expression in the intact human heart. Downregulation of alpha-myosin heavy chain in hypertrophied, failing ventricular myocardium, *J Clin Invest.* 1997 Nov;100(9):2315- 2324. <https://doi.org/10.1172/JCI119770>
30. Miyata S, Minobe W, Bristow MR, Leinwald LA. Myosin heavy chain isoform expression in the failing and nonfailing human heart. *Circ Res.* 2000 Mar;86(4):386-90. <https://doi.org/10.1161/01.res.86.4.386>.
31. Perez S, Royo LJ, Astudillo A, Escudero D, Avarez F, Rodriguez A, et al. Identifying the most suitable endogenous control for determining gene expression in hearts from organ donors. *BMC Mol Biol.* 2007 Dec;8:114. <https://doi.org/10.1186/1471-2199-8-114>
32. Pilbrow AP, Ellmers LJ, Black MA, Moravec CS, Sweet WE, Troughton RW, et al. Genomic selection of reference genes for real-time PCR in human myocardium. *BMC Med Genomics* 2008 Dec;1:64. <https://doi.org/10.1186/1755-8794-1-64>
33. You L, Xie R, Hu H, Gu G, Zheng H, Zhang J, et al. High levels of serum β 2-microglobulin predict severity of coronary artery disease. *BMC Cardiovasc Disord.* 2017 Mar;17(1):71. <https://doi.org/10.1186/s12872-017-0502-9>.
34. Vianello A, Caponi L, Galetta F, Franzoni F, Taddei M, Rossi M, et al. β 2-microglobulin and TIMP1 are linked together in cardiorenal remodeling and failure. *Cardiorenal Med* 2015 Feb;5(1):1-11. <https://doi.org/10.1159/000369260>
35. Molenaar B, Timmer LT, Droog M, Perini I, Versteeg D, Kooijman L, et al. Single-cell transcriptomics following ischemic injury identifies a role for B2M in cardiac repair. *Commun Biol.* 2021 Jan;4(1):146. <https://doi.org/10.1038/s42003-020-01636-3>.
36. Pollard TD, Cooper JA. Actin, a central player in cell shape and movement. *Science* 2009 Nov;326(5957):1208–1212. <https://doi.org/10.1126/science.1175862>
37. Heling A, Zimmermann R, Kostin S, Maeno Y, Hein S, Devaux B, et al. Increased expression of cytoskeletal, linkage, and extracellular proteins in failing human myocardium. *Circ Res* 2000 Apr;86(8):846–853. <https://doi.org/10.1161/01.res.86.8.846>
38. Bunnell TM, Burbach BJ, Shimizu Y, Ervasti. β -Actin specifically controls cell growth, migration, and the G-actin pool. *Mol Biol Cell* 2011 Nov;22(21):4047-4058. <https://doi.org/10.1091/mbc.E11-06-0582>

39. Serebryanny LA, Parilla M, Annibale P, Cruz CM, Laster K, Gratton E, et al. Persistent nuclear actin filaments inhibit transcription by RNA polymerase II. *J Cell Sci.* 2016 Sep;129(18):3412–3425. <https://doi.org/10.1242/jcs.195867>
40. Xing W, Zhang TC, Cao D, Wang Z, Antos CL, Li S, et al. Myocardin induces cardiomyocyte hypertrophy. *Circ Res.* 2006 Apr;98(8):1089-1097. <https://doi.org/10.1161/01.RES.0000218781.23144.3e>
41. Wesolowska N, Lénárt P. Nuclear roles for actin. *Chromosoma* 2015 Dec;124(4):481-489. <https://doi.org/10.1007/s00412-015-0519-8>
42. Kondrikov D, Fonseca FV, Elms S, Fulton D, Black SM, Block ER, et al. β -Actin association with endothelial nitric-oxide synthase modulates nitric oxide and superoxide generation from the enzyme. *J Biol Chem.* 2010 Feb;285(7):4319–4327. <https://doi.org/10.1074/jbc.M109.063172>

Tables

Table 1 Clinical, echocardiographic and biochemical parameters prior to transplantation.

Total number of patients		28*
Sex	Male	25
	Female	3
Age		54.4±8.2
Systolic blood pressure - SBP [mmHg]		112±18
Diastolic blood pressure – DBP [mmHg]		67±11
Heart rate [min ⁻¹]		79±16
Etiology	Dilated Cardiomyopathy – (DCM)	10
	Coronary Artery Disease – (CAD)	9
	Hypertrophic cardiomyopathy – (HCM)	4
	Ischemic cardiomyopathy – (ICM)	5
Echo	Left ventricular ejection fraction [%]	27 ± 14
	Left ventricular end-diastolic diameter [mm]	68.8 ± 15.8
	Right ventricular end-diastolic diameter [mm]	32.8 ± 5.1
	Interventricular septal end-diastolic [mm]	9.4 ± 2.5
ECG	QRS complex [s]	0.140 ± 0.05
	QT interval [s]	0.42 ± 0.04
Biochemical parameters	NT-proBNP [ng/l]	5551 ± 4259
	Glucose [nmol/l]	7.13 ± 3.4
	Uric acid [μmol/l]	500 ± 192
	Total cholesterol [mmol/l]	4.15 ± 1.08
	Triacylglycerol [mmol/l]	1.56 ± 0.8
	Sodium [mmol/l]	136.3 ± 4.6
	Potassium [mmol/l]	4.4 ± 0.39
	Creatine kinase [μkat/l]	3.54 ± 6.4
	Urea [mmol/l]	9.02 ± 4.3
	Creatinine [μmol/l]	112 ± 35

* data are expressed as mean ±S.D.

Table 2 Stability values and significance levels of CRGs based on ANOVA analysis. In bold, $p \leq 0.05$.

Gene	Variance component		F	Significance	V_B	V_W	Stability index	Ranking
	between groups	within group						
IPO8	0,002	0,061	0,037	0,848	0,004	0,107	0,00042	1
PGK1	0,013	0,054	0,232	0,634	0,036	0,155	0,006	2
HPRT1	0,104	0,053	1,957	0,172	0,203	0,104	0,021	3
GAPDH	0,134	0,059	2,276	0,142	0,243	0,107	0,026	4
YWHAZ	0,623	0,041	15,254	0,0004	0,775	0,051	0,039	5
POLR2A	0,225	0,057	3,928	0,056	0,408	0,104	0,042	6
PPIA	0,275	0,143	1,920	0,176	0,339	0,177	0,060	7
TBP	0,244	0,031	7,883	0,009	0,695	0,088	0,061	8
TRFC	0,448	0,212	2,114	0,156	0,364	0,172	0,063	9
UBC	0,208	0,091	2,283	0,141	0,386	0,169	0,065	10
HMBS	0,735	0,213	3,450	0,073	0,654	0,190	0,124	11
GUSB	0,472	0,121	3,908	0,057	0,757	0,194	0,147	12
RPLP0	2,356	0,123	19,167	<0,0001	1,959	0,102	0,200	13
B2M	2,487	0,093	26,871	<0,0001	2,499	0,093	0,232	14
ACTB	3,278	0,114	28,636	<0,0001	2,637	0,092	0,243	15

Table 3 Final ranking of the candidate reference genes.

Ranking	ANOVA	geNorm	NormFinder	BestKeeper	Delta Cq	Final ranking*
1	IPO8	POLR2A	IPO8	IPO8	IPO8	IPO8
2	PGK1	IPO8	PGK1	PPIA	POLR2A	POLR2A
3	HPRT1	GAPDH	HPRT1	POLR2A	UBC	PGK1
4	GAPDH	UBC	UBC	HMBS	PGK1	UBC
5	POLR2A	PGK1	GUSB	UBC	GAPDH	HPRT1
6	PPIA	HPRT1	PPIA	GUSB	HPRT1	GAPDH
7	TFRC	PPIA	GAPDH	TFRC	PPIA	PPIA
8	UBC	GUSB	POLR2A	GAPDH	GUSB	GUSB
9	HMBS	HMBS	TFRC	PGK1	HMBS	HMBS
10	GUSB	TFRC	HMBS	HPRT1	TFRC	TFRC

* Final raking was calculated as a geometric mean of ranking positions from all five methods, the three most stably expressed genes are shown in bold.

Figures

Figure 1

Individual ranking lists based on the geNorm, NormFinder, Delta Cq and BestKeeper analyses of CRGs expression. The coloured bars represent the indexes of stability (M-value, Stability value, Mean SD, Correlation coefficient to BestKeeper index, respectively) calculated in each analysis. The ranking position of each gene is indicated.

Figure 2

Relative expression of MYH 6 and MYH 7 genes in LV of healthy and failing human hearts. Plot represents the mean $\pm$ SEM gene expression ratio of both genes normalized to IPO8, PGK1 and POLR2A. ** indicate $p \leq 0.01$.

Figure 3

Relative expression of ACTB and B2M genes in LV of healthy and failing human hearts. Plot represents the mean $\pm$ SEM gene expression ratio of both genes normalized to IPO8, PGK1 and POLR2A. *** indicate $p \leq 0.001$.

Supplementary Files

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- [SupplementarymaterialSelectionofRGsforRTqPCR.docx](#)