Normalization of qPCR in platelets – YWHAE a potential generic reference gene

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Abstract

The mRNA of human platelets has been extensively studied and it is generally appreciated that platelets contain mRNA transcripts derived from the megakaryocytes, and they have the ability to translate it into proteins. Additionally, platelets contain microRNA (miRNA) that has been shown to potentially regulate the translation of certain proteins. When quantifying gene expression by quantitative real-time polymerase chain reaction (qPCR), a valid normalization method is required and the use of reference genes is a common and robust approach. It is recommended to perform a proper validation of potential reference genes for each individual experimental setup. Previous studies have mainly been performed using commonly used reference genes for nucleated cells, and to our knowledge there are no global evaluations of the stability of transcripts in platelets. Finding a stable transcript would be valuable for inter-study comparisons, and the aim of this study was to identify one or more stable mRNA transcripts suitable as generic reference genes for mRNA gene expression studies in platelets. Platelets were incubated for 24 h and microarray of platelet mRNA revealed that the levels of YWHAE, B2M, ITM2B, H3F3A, PF4V1 remained similar between 0 and 24 h. Further validation of the stability of these genes together with GAPDH, RNI18S1, and PPIA, genes frequently used as reference genes in platelet studies, was performed using qPCR after different in vitro conditions. In addition, inter-individual stability of the genes was analyzed in diabetic patients compared with healthy matched controls. Analysis of gene stability by the software RefFinder revealed that YWHAE, PFF4V1, and B2M were the most stable genes in platelets from healthy donors. In addition, YWHAE was stable between subjects. Furthermore, the potential influence of miRNA on the selected genes was investigated by knockdown of Dicer1 in the megakaryocytic cell line MEG01. YWHAE, H3F3A, B2M, and GAPDH remained unchanged over time in MEG01 cells indicating that these genes are not regulated by miRNA and hence are more stably expressed. In conclusion, YWHAE is a stable transcript in platelets and we suggest the use of YWHAE as a generic reference gene in mRNA gene expression studies.

Keywords

miRNA, mRNA, normalization, platelets, qPCR, reference genes

Introduction

The nature of platelet mRNAs has been widely studied in the last decade, as reviewed in [1]. It is known that platelets not only contain mRNA but also the machinery to translate it into proteins [2–4]. Platelets express some 2300 mRNA transcripts which, predictably, are derived from megakaryocytes [5,6]. There is even some evidence of active sorting of transcripts into platelets by megakaryocytes [7]. Platelets are also the only known cells that can splice pre-mRNA into mature mRNA with a functional spliceosome in the cytoplasm [4]. Furthermore, human platelets contain microRNA (miRNA), which are a class of ~22 nucleotide RNAs, that regulate protein-coding genes primarily by repressing gene expression either by mRNA degradation or translational repression [8–13]. In addition to functional miRNA transcripts, platelets also contain the machinery to process pre-miRNA into mature miRNA [14,15].

Quantitative real-time polymerase chain reaction (qPCR) is a robust and commonly used method to quantify gene expression [16]. However, the importance of a solid and validated normalization strategy in order to receive accurate results should not be underestimated. Potential errors can occur at various stages of qPCR, of which the most common are when obtaining the material, during the RNA extraction, the quality and storage of the RNA, the cDNA synthesis, the selection of primer and probe sets, and during the statistical analysis [17]. Theoretically, an identical volume of tissue/cells or an identical amount of total mRNA could be used as normalization strategy. However, in practice such strategies will induce too much variability for most applications [18–20]. To date, the use of internal control genes, referred to as reference genes, is the most effective method to correct for errors, since all stages to receive the final PCR product are controlled for [18,21]. To be considered a reliable reference gene, it has to be validated [19]. It has been shown that commonly used reference genes are not necessarily the most stable [22], and importantly, using the wrong reference gene may end in altered

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findings [23]. Therefore, it is recommended to validate reference genes for each individual experimental setup. There are several web-based programs that can be used to identify the most stable genes among a set of candidate genes in an experiment of interest. GeNorm calculates the geometric mean of the gene expression of data converted to copy number to select the least variable gene [21]. A second program, BestKeeper, uses the same method as GeNorm, but using the raw data [24]. NormFinder measures the variation and ranks the reference genes based on the impact of the experimental conditions between observations [25].

This study aimed to characterize stable reference genes for qPCR in platelets and, if possible, identify a single gene or alternatively a small set of genes that could be used as a generic internal reference for platelet mRNA expression studies. We used a microarray approach in order to identify stable genes, and the stability was further investigated in platelets from healthy donors incubated in different conditions. Platelets from subjects with type 2 diabetes (T2D) and controls were investigated regarding intra-individual stability, since diabetic platelets are assumed to be hyper-reactive [26]. Additionally, miRNAs complimentary to the selected candidate genes were studied to elucidate if the miRNAs are targets of miRNA repression, which may influence stability of the candidate genes.

Materials and methods

Protocols were approved by the ethics committee of the University of Gothenburg, Sweden.

Platelet isolation from healthy blood donors

Platelets were collected from blood donors at the Blood Center, Sahlgrenska University Hospital, Gothenburg, Sweden. Platelet-rich Buffy coat was produced from whole blood by component manufacture, where after four Buffy coats were pooled in platelet medium (NaCitrate, NaCl, NaAc, pH 7.2). One pool contained platelets from a total of four donors. Platelet concentration was 515 – 905 x 10⁹/L. Leukocytes were eliminated by filtration; the contamination rate was 1 leukocyte per 25 million platelets at the most.

Microarray hybridization and selection of candidate genes

Total RNA was extracted from half of each of the two platelet pools at time 0 h and the remaining platelets were incubated for 24 h on a rotary shaker at 22°C before RNA extraction using Chomzynski/Sacci method, described elsewhere [27], was done with MaXtract High Density Tubes (50 ml; Qiagen GmbH, Hilden, Germany). RNA was resolved in RNAse-free H2O. To improve the quality and purity of RNA, RNase MinElute Cleanup kit was used according to manufacturer’s instruction (Qiagen). RNA quality was analyzed on 1% agarose gel, and RNA quantity was analyzed using a spectrophotometer (A260/A280). Total RNA was hybridized to a Human Genome U133 Plus 2.0 chip that was analyzed using a spectrophotometer (A260). Total RNA from all platelets was extracted using Trizol according to manufacturer’s instructions (Ambion by Life Technologies, Paisley, Scotland, UK). Total RNA concentrations were determined on Qubit 2.0 Fluorometer using Qubit RNA HS Assay Kit according to manufacturer’s instructions (Life Technologies, Eugene, OR, USA) and the levels were similar. Reverse transcription was carried out in a fixed volume of RNA in a total volume of 20 µl reaction mixture, 1x RNA-to-cDNA master mix (Applied Biosystems, Foster City, CA, USA). Samples were incubated at 25°C for 5 min, at 42°C for 30 min, 85°C for 5 min, and finally at 4°C. Relative quantification was performed on 7500 Fast Real-Time PCR System (Applied Biosystems). For amplification of the candidate genes, 0.24 µl cDNA was added to the PCR mixture consisting of TaqMan Universal PCR Master Mix Fast, in a final volume of 15 µl (Applied Biosystems). Primers and probes of GAPDH and PAI-1 were designed using the Primer Express, version 1.0, software (Applied Biosystems) and were used in a concentration of 0.4 and 0.2 µM, respectively. Each primer pair was selected so that the amplicon spanned an exon junction to preclude amplification of genomic DNA. The efficiencies of the in-house-designed primer and probes were validated by using a dilution curve (data not shown). Pre-designed primers and TaqMan probes (Gene Expression Assays, Applied Biosystems) for PPIA, RN18S1, YWHAE, B2M, ITM2B, H3F3A, and PF4V1 were used (Table I).

Table I. Primers and probes used for qPCR.

| Gene symbol | Sequence | Position | Gene expression assay |
|-------------|----------|----------|-----------------------|
| YWHAE      |          |          | Hs00356749_g1         |
| B2M        |          |          | Hs00187842_m1         |
| ITM2B      |          |          | Hs00222753_m1         |
| H3F3A      |          |          | Hs02598544_g1         |
| PF4V1      |          |          | Hs0060249_g1          |
| RN18S1     |          |          | Hs99999901_s1         |
| PPIA       |          |          | Hs041945421_s1        |

Quantitative reverse transcriptase real-time PCR of platelets from healthy donors

Pooled platelets were either activated or inhibited. Activated platelets were initially centrifuged (15 min at 800g) and resuspended in pipperazine diethanesulfonic acid (PIPES)/saline/glucose (5 mM PIPES, 145 mM NaCl, 4 mM KCl, 50 µM Na₂HPO₄, 1 mM MgCl₂, and 5.5 mM glucose) containing 100 nM prostaglandin E₁ (PGE₁) (Sigma, St. Louis, MO, USA). After centrifugation (15 min at 2000g) platelets were incubated in M199 without phenol red (Sigma) for 0, 24, and 48 h in three different activation conditions; initially inhibited by 100 nM PGE₁ and activated after 6 h, activated by 25 µM PAR receptor agonist SFLLRN (Bachem, Bubendorf, Switzerland) or in M199 without phenol red only. Inhibited platelets were kept inhibited in medium (NaCitrate, NaCl, NaAc, pH 7.2) from the Blood Center for 0, 24, and 48 h. Total RNA from all platelets was extracted using Trizol according to manufacturer’s instructions (Ambion by Life Technologies, Paisley, Scotland, UK).

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Quantitative reverse transcriptase real-time PCR of platelets from subjects with T2D and controls

Platelet isolation and RNA extraction was performed as previously described [28]. Reverse transcription was carried out in 0.5 μg of RNA in a total volume of 20 μl reaction mixture, using high-capacity RNA-to-cDNA kit (Applied Biosystems). Samples were incubated at 25°C for 5 min, at 42°C for 30 min, 85°C for 5 min, and finally at 4°C. Relative quantification was performed on 7500 Fast Real-Time PCR System (Applied Biosystem). For amplification of the candidate genes 0.24 μl cDNA was added to the PCR mixture consisting of TaqMan Universal PCR Master Mix Fast (Applied Biosystems), in a final volume of 15 μl. Primer and probes were used as stated above.

miRNA method

The megakaryocyte cell line MEG-01 were transfected with 30 nM of Dicer siRNA or scrambled negative control siRNA using nucleofection (Lonza, Basel, Switzerland). After 72 h transfection, cells were harvested and RNA prepared using the miRNeasy mini kit (Qiagen). cDNA was prepared using the RevertAid H-First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) with 1 μg of RNA per reaction. qPCR was run using TaqMan assays on a StepOnePlus instrument (Thermo Fisher Scientific). Results are from three separate experiments, with triplicates in each group.

Precision validation

To establish the precision of the stable genes found, plasminogen activator inhibitor 1 (PAI-1) (Table 2), a mRNA transcript that is known to be abundant in platelets [29], was used as reference mRNA. The cohort consisted of platelets from eight healthy volunteers. Replicate measurements by qPCR were tested, as previously described [30].

Data analysis

In total, four microarrays (two pools of donors) were analyzed before and after 24 h incubation, and scanned output files from the microarray were visually inspected for hybridization artifacts before and after 24 h incubation, and scanned output files from the microarray were visually inspected for hybridization artifacts. Five genes, YWHAE, PF4V1, B2M, ITM2B, and GAPDH, RN18S1, and PPIA were chosen as candidates for analysis since they have previously been used as reference genes in studies of platelets [29,33–37]. In total, these eight genes were selected as candidate genes for further characterization (Table 3).

Expression of candidate genes in platelets from healthy donors

All candidate genes had raw Ct values ranging from 15 to 33. Stability, identified as geometric means by RefFinder, of the candidate reference genes in platelets are shown in Table 3. At least three genes should be used as reference genes for a correct normalization according to previous findings [21]. YWHAE, PF4V1, and B2M were most stably expressed in the total material where all experimental data were used in combination. In activated platelets, YWHAE, PF4V1, and B2M were the most stably expressed genes, as seen in the total material. There was no difference in stability between the three various conditions in which the platelets were activated (data not shown). Inhibited platelets showed a different result, where ITM2B was most stably expressed followed by PF4V1, B2M, and YWHAE. Platelets were incubated for 0, 24, and 48 h. Initially, ITM2B was most stably expressed, followed by YWHAE, B2M, and PF4V1. However, after 24 and 48 h PF4V1, YWHAE, and B2M were the most stably expressed genes. In summary, YWHAE, PF4V1, and B2M are most stably expressed genes in platelets from healthy donors. In inhibited non-incubated platelets, ITM2B is most stably expressed. However, this candidate gene is not stable over time and across different types of
incubations. GAPDH was consistently never ranked as the most stable gene, and never the least stable gene. PPIA and RN18S1 were the least stable genes.

Expression of candidate genes in platelets from donors with disease/control

In order to elucidate if there are inter-individual differences, five subjects with type 2 diabetes (T2D) and five gender- and age-matched controls were studied (Table 4). In both groups YWHAE was the most stable gene followed by H3F3A and ITM2B.

MicroRNA

To investigate if miRNAs affect the stability of the candidate genes, the megakaryocyte cell line MEG-01 was transfected with Dicer1 siRNA. After 72 h transfection, Dicer1 expression was decreased by 61.7\% (P = 0.000008) compared to cells transfected with scrambled control siRNA. Moreover, a number of highly abundant platelet miRNAs were depleted in cells transfected with Dicer1 siRNA, confirming that Dicer1 knockdown led to disrupted miRNA biogenesis (Supplementary Figure 1). Global mean normalization was used to normalize for each of the candidate genes. A significant increase compared with control cells was seen for ITM2B and PPIA, by 26.0\% (P = 0.009) and 25.3\% (P = 0.019), respectively (Figure 1). RN18S1 was increased by 24.5\%, but this was non-significant (P = 0.064). YWHAE, H3F3A, B2M, and GAPDH remained unchanged, whereas PF4V1 was not detectable at all.

Precision validation

YWHAE and GAPDH were normalized to PAI-1. There was a higher correlation of reference PAI-1 mRNA expression between the first and the second run after normalization for YWHAE than for GAPDH and the correlation for YWHAE was statistically significant (r = 0.761, P = 0.001; and r = 0.349, P = 0.186, respectively). This confirms that the precision of YWHAE is superior to that of GAPDH.

Discussion

This study demonstrates that YWHAE, PF4V1, and B2M were the three most stable genes in platelets from healthy donors irrespective of degradation time or activation/inhibition of the platelets. Although frequently used, GAPDH, RN18S1, and PPIA were relatively unstable genes and consequently not suitable as generic reference genes. In platelets from subjects with T2D, YWHAE was the most stable reference gene.

Table III. Comprehensive gene stability by RefFinder in platelets from healthy donors.

|        | YWHAE | PF4V1 | B2M | ITM2B | H3F3A | GAPDH | PPIA | RN18S1 |
|--------|-------|-------|-----|-------|-------|-------|------|--------|
| All data | 1,565 | 1,861 | 2,943 | 6,236 | 3,722 | 3,162 | 7,238 | 7,445  |
| Activated | 1,565 | 1,861 | 2,943 | 6     | 3,722 | 3,162 | 7,238 | 7,737  |
| Inhibited | 2,828 | 2     | 2,28  | 1,778 | 4,821 | 5,477 | 7     | 8      |
| Non-incubated | 1,861 | 3,364 | 2,449 | 1,316 | 5,477 | 5,477 | 7     | 8      |
| Incubated 24 h | 1,861 | 1,565 | 2,913 | 5,433 | 4,472 | 2,943 | 7     | 8      |
| Incubated 48 h | 1,861 | 2,060 | 2,236 | 6,481 | 4     | 2,943 | 8     | 6,481  |

Platelets are pooled from four healthy donors in three different experiments. Isolated platelets are incubated under different in vitro conditions. The three most stable genes are marked in bold for each in vitro experiment. See abbreviations of gene symbols in Table II.

Table IV. Comprehensive gene stability by RefFinder in platelets from T2D and controls.

|        | YWHAE | PF4V1 | B2M | ITM2B | H3F3A | GAPDH | PPIA | RN18S1 |
|--------|-------|-------|-----|-------|-------|-------|------|--------|
| T2D | 1,32  | 7,44  | 4,47 | 3,13  | 1,41  | 3,87  | 7    | 6,45   |
| non-T2D | 1,41  | 7     | 5,23 | 2,06  | 1,73  | 4     | 8    | 5,73   |

Platelets are isolated from five subjects with type 2 diabetes and five age- and gender-matched healthy control subjects. The three most stable genes are marked in bold for each experiment. T2D, type 2 diabetes, see abbreviations of gene symbols in Table II.
The most common way researchers select reference genes is by literature searches or by simply selecting a commonly used reference gene. However, traditional reference genes were reported as early as the 1970s to be differently expressed in tissues, and despite these observations they have been used extensively, without proper validation, in published studies [38,39]. In order to perform an unbiased search for genes that are stable over time, a microarray experiment was performed. In this experiment, YWHAE, B2M, ITM2B, H3F3A, P4F1V1 were the five most stable genes in platelets over time, even though none of these has previously been reported as a suitable reference gene in platelets. However, there are potential pitfalls in using microarray in this experimental setting. First, platelets used for the microarray were inhibited until RNA isolation, and hence does not include the whole experimental conditions used for the qPCR evaluation of the reference genes. Second, one could also argue that no gene from the microarray experiment is an excellent reference gene, since no gene was completely stable over time as the signal from all genes decreased over time. The most likely explanation is that the mRNA content in platelets, derived from the megakaryocytes, is degraded over time despite inhibition of the platelets. Even with these potential pitfalls, the unbiased approach using microarray for selection of candidates for validation is preferable to choosing candidate reference genes from the literature since it opens up for discovery of genes not previously considered as reference genes. The validity of this approach is indicated in the results showing that the stability of the genes chosen from the microarray experiment in general was higher than the genes selected from the literature.

There are several methods to validate reference genes for qPCR. In this study the software RefFinder was used, since it integrates four different approaches: GeNorm, NormFinder, BestKeeper, and \(\Delta \Delta CT\) method. RefFinder uses raw CT data and to adjust for the efficiencies of the qPCR assays used, GeNorm and NormFinder were evaluated separately to take the efficiencies into account [40]. The result was the same, independent of the method used. In order to evaluate the possible effect of miRNA on the stability of the selected reference genes, we performed a thorough review of the literature but could not find any evidence that any of the candidate genes are targeted by miRNAs. Dicer1 is required for miRNA biogenesis by cleaving double-stranded pre-miRNA [41]. In this study, YWHAE, B2M, H3F3A, and GAPDH were not influenced by Dicer1 knockdown and is therefore not expected to be regulated by miRNAs, a result in agreement with the result from RefFinder analysis. ITM2B, and PPIA, increased in MEG01 cells after Dicer1 knockdown, which may indicate derepression of gene expression as a result of decreased miRNA levels.

There was some variation in stability of the candidate genes but YWHAE was the most stable gene throughout all experimental conditions and was not influenced by miRNA. B2M, GAPDH, and H3F3A varied in stability; however, they were not under miRNA regulation and may well be used as candidates when validating reference genes in platelets. On the other hand, ITM2B, PPIA, and RN18S1 were unstable and potentially regulated by miRNA and are therefore not robust candidates. GAPDH was not among the most stable genes, nevertheless it appeared not to be influenced by miRNA, and since it is widely used as a reference gene in platelets it may be considered for further research if it is desired to compare results with previous studies. Additionally, validation of precision of the candidate genes in this study revealed that the precision of YWHAE was superior to GAPDH, which further indicates that YWHAE is a stable reference gene in platelets. To our knowledge, only one previous study has investigated reference genes in platelets, in this study platelets from healthy donors and subjects with a history of myocardial infarction were used [42]. GAPDH was the most stable gene in healthy donors (geometric mean 2.34), however, except for GAPDH, a comparison of results is not applicable, since different candidate genes were used.

In conclusion, this study illustrates the importance of carefully selecting reference genes for each set of experiment when using qPCR. We show for the first time, that YWHAE is a stable reference gene across different experimental conditions and intra-individual variations in platelets. We suggest YWHAE to be considered as a potential candidate internal control gene when evaluating reference genes for any experimental setup in platelets.

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**Declaration of interest**

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**Supplemental data**

Supplemental data for this article can be accessed on the publisher’s website.

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