Selection of Suitable Housekeeping Genes for Real-Time Quantitative PCR in CD4⁺ Lymphocytes from Asthmatics with or without Depression

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Abstract

Objective: No optimal housekeeping genes (HKGs) have been identified for CD4⁺ T cells from non-depressive asthmatic and depressive asthmatic adults for normalizing quantitative real-time PCR (qPCR) assays. The aim of present study was to select appropriate HKGs for gene expression analysis in purified CD4⁺ T cells from these asthmatics.

Methods: Three groups of subjects (Non-depressive asthmatic, NDA, n = 10, Depressive asthmatic, DA, n = 11, and Healthy control, HC, n = 10 respectively) were studied. qPCR for 9 potential HKGs, namely RNA, 28S ribosomal 1 (RN28S1), ribosomal protein, large, P0 (RPLP0), actin, beta (ACTB), cyclophilin A (PPIA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1 (PGK1), beta-2-microglobulin (B2M), glucuronidase, beta (GUSB) and ribosomal protein L13a (RPL13A), was performed. Then the data were analyzed with three different applications namely BestKeeper, geNorm, and NormFinder.

Results: The analysis of gene expression data identified B2M and RPLP0 as the most stable reference genes and showed that the level of PPIA was significantly different among subjects of three groups when the two best HKGs identified were applied. Post-hoc analysis by Student-Newman-Keuls correction shows that depressive asthmatics and non-depressive asthmatics exhibited lower expression level of PPIA than healthy controls (p<0.05).

Conclusions: B2M and RPLP0 were identified as the most optimal HKGs in gene expression studies involving human blood CD4⁺ T cells derived from normal, depressive asthmatics and non-depressive asthmatics. The suitability of using the PPIA gene as the HKG for such studies was questioned due to its low expression in asthmatics.

Introduction

It has been proposed that a spectrum of psychological conditions such as depressive disorders occurs at high frequencies in asthmatics [1], and are associated with poor control and worse asthma-related quality of life [2], but the underlying pathophysiological mechanisms that account for this relationship have yet to be elucidated [3]. Since the initial studies of the roles of T cells in the pathogenesis of asthma [4,5], our understanding of the CD4⁺ T lymphocyte in the immunopathology of this disease has greatly advanced over the past decades, involving not only the classic Th1 and Th2 cells, but also new proinflammatory and suppressive T-cell subsets [6]. Meanwhile, accumulating evidence suggests that CD4⁺ T cells may influence susceptibility to depression as well as its treatment outcomes [7]. Thus, the CD4⁺ T lymphocyte is emerging as a potentially attractive cell in which to seek novel insights into the pathogenesis of asthma with or without depression and to identify new therapeutic targets.

The comparison of gene expression profiling of CD4⁺ T cells in asthmatic subjects with and without depressive disorders can lead to the identification of genes implicated in such diseases and provide added insight into the underlying pathophysiological mechanisms. Real-time quantitative PCR (qPCR) is a useful technique for acquiring the gene expression pattern of a number of selected genes due to its high sensitivity, specificity and broad quantification range [8]. To obtain accurate and reliable gene expression quantification, normalization of gene expression data against housekeeping genes (HKGs) is particularly important. For this purpose, an ideal HKG should be either stably expressed across experimental conditions or similarly expressed among samples affected by different disease processes. However, commonly used HKGs vary considerably in different disease processes or different tissue and cell types. Thus, it is important to perform

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The mean ± SD number of total lymphocytes was 22.87 ± 5.68 million. After separation, each sample yielded ~2.4 million CD4⁺ T cells and 1 million cells were used for RNA extraction from each sample. Furthermore, our pilot studies have confirmed that the CD4⁺ T cell population isolated by this method has a purity of over 94%, which was shown by flow cytometry (Figure 1).

Selection of Reference Gene Candidates

Eleven HKGs from the endogenous control panel genes recommended by Applied Biosystems [http://www.appliedbiosystems.com/] were initially selected. 18 S ribosomal RNA (RNR1) was replaced by RNA, 28 S ribosomal 1 (RN28S1) due to their stable expression ratio in integrated RNA samples and the availability of RN28S1 assay in our laboratory. Ribosomal protein L13a (RPL13A) was added because it was a stable
expression gene in CD4+ cells from a previous study [12]. Among the 12 genes selected, hypoxanthine ribosyltransferase (HRYT), TATA-binding protein (TBP) and transferrin receptor (TFRC) have low expression levels in the CD4+ cells and whole blood therefore they were omitted from the final list (http://www.genecards.org/).

Nine housekeeping genes were examined, including RN28S1, ribosomal protein, large, P0 (RPLP0), actin, beta (ACTB), cyclophilin A (PPIA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1 (PGK1), beta-2-microglobulin (B2M), glucuronidase, beta (GUSB), and RPL13A. The full name, function and accession number of the candidate HKGs evaluated in the present study are listed in Table 3. Special attention was paid to selecting candidate genes that show a diversity of function, which significantly reduces the chance that genes might be co-regulated.

RNA Extraction and Complementary DNA Preparation

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, California, USA) following the manufacturer’s protocol. RNA integrity was assessed on the basis of demonstration of distinct 28 s and 18 s ribosomal RNA bands following 1% agarose electrophoresis and the 28 s RNA was approximately twice as intense as the 18 s RNA. Complementary DNA (cDNA) synthesis was carried out using the RevertAid™ first strand cDNA synthesis kit (Fermentas Inc, Burlington, Canada). Template RNA and 1 μL of random hexamer primers (10 μM) in a total volume of 12 μL were incubated for 5 min at 65°C and chilled on ice. After adding 4 μL of 5× reaction buffer, 1 μL of Ribolock™ RNase Inhibitor (20 U/μL), 2 μL of dNTP Mix (10 mM), 1 μL of RevertAid™ M-MuLV Reverse Transcriptase (200 U/μL), the incubation step for 5 min at 25°C, followed by reverse transcriptase incubation for 60 min at 42°C, termination of the reaction by heating at 70°C for 5 min, finally cooling to 4°C before storage at −20°C. The cDNA for assays of ACTB, GAPDH, B2M, PPIA and RPLP0 was diluted 1:25 because these genes were highly expressed in pilot studies; while assays of RN28S1, GUSB, RPL13A and PGK1 were performed using cDNA diluted 1:15 because they had relatively low expression levels.

Real-time Quantitative PCR

The expression analysis for all 9 genes was performed using an FTC 2000 qPCR system (Funglyn Biotech Inc, Scarborough, Canada). PCR primers and TaqMan probes were obtained from Shanghai biological engineering corporation, China (see Table 4 for primer sequences). The reactions were performed according to the manufacturer’s instructions with minor modifications. Briefly, 2 μL template cDNA was used in a final PCR reaction volume of 30 μL, containing 0.3 μL of 5 U/μL Taq DNA polymerase, 3 μL of 25 mM MgCl2, 0.36 μL of 25 mM dNTP, (TAKARA Bio Group, Dalian, China), 1 μL of 10 μM of each forward and reverse primer and probe. The conditions for the PCR included for 2 min at 94°C followed by 45 cycles of real-time PCR with 3-segment amplification, including 20 s at 94°C for denaturation, 20 s at 52°C (RN28S1, PPIA, GAPDH and RPL13A), 54°C (RPLP0, ACTB, GUSB) or 56°C (PGK1 and B2M) for annealing, and 30 s at 60°C for polymerase elongation. All reactions were performed in triplicate, with non-template controls and standard curves which were generated using four serial dilution points (in steps of 10-fold) of stock cDNAs for each gene. The threshold cycle (Ct) was manually determined from amplification plots. The ΔCt value for each sample was obtained by subtracting the Ct values of the highest relative quantities for each gene, and was converted into relative gene expression by the amplification efficiency (2^(-ΔCt)) to the −ΔCt power.

Statistical Analysis

In order to identify the optimal reference genes among the candidates, three different tools called BestKeeper, geNorm, and NormFinder based on specific algorithms were used. The BestKeeper [13] and geNorm [14] determines the optimal HKGs by performing similar pair-wise correlation approach. The NormFinder produces a comparison of the rankings by a model-based approach and focuses on estimating both the overall variation of the reference genes and the variation between subgroups [15].

Clinical data are reported as mean ± SD for normally distributed data and median (range) for nonparametric data. Descriptive statistics of the 8 HKGs were computed by BestKeeper.

The comparisons of gene expression levels and demographic characteristics of the participants between subgroups were performed by using the one-way ANOVA (two tailed) for parametric data, Kruskal-Wallis H test for nonparametric data and Student-Newman-Keuls test for multiple comparisons. All analyses were conducted with SPSS software, version 18.0 (IBM Corp, New York, USA). P<0.05 was considered significant.

Results

Subjects

Characteristics of the three groups of participants are summarized in Table 1. By design, all 3 groups (NDA, n = 1, DA, n = 11, and HC, n = 10) were similar in sex and age distribution. All subjects were non-smokers or former smokers and there were only two former smokers, one in NDA group and the other in NC group. Both of them have quitted at least 10 years and had smoked cigarette 4.5 and 0.5 pack-years, respectively. All medications were discontinued for a minimum of 2 weeks before recruitment. In detail, one patient from the NDA group and two from DA group inhaled inhaled corticosteroid (ICS) + Long-acting β2-agonists (LABA). However, all of them used ICS + LABA for a maximum of 1 month and discontinued at least 4 weeks before blood was drawn. Four patients from the NDA group and three from the DA group took theophylline, and one patient from each group took antieukotrienes orally. However, the medications were discontinued at least 2 weeks before the experiment.

There were significant differences between subgroups in FEV1% predicted, FEV1/forced vital capacity (FVC) %, the proportion having anaphylactic history and total immunoglobulin E (IgE) present in each sample. Age, sex, body mass index (BMI), the proportion of participants who were atopic, number of eosinophils and the proportion of eosinophils did not differ among the three groups. There were no significant differences in demographic characteristics such as PD20FEV1 and asthma severity, etc. between NDA and DA groups (see Table 1 for detail).

Expression Levels of Candidate HKGs

Nine HKGs (n = 31) were investigated and triplicate assays were performed for each of the 31 subjects. The Ct values were over 45 for 5 samples for GAPDH and therefore this gene was excluded from further analysis. For the remaining 8 HKGs, two samples had three unidentified Ct values in assays for two genes: one sample from the HC group and the other from the DA group. Thus both of these samples were excluded from further analysis. One of the specimens investigated exhibited 3-fold intrinsic variance (InVar) compared with the mean InVar of all HKGs,
therefore, this subject was excluded. The final analysis contained 28 samples for 8 HKGs (Table 2).

Expression Stability within HKGs

HKG stability was evaluated using three different Excel-based tools, BestKeeper, geNorm and NormFinder. The BestKeeper was used to rank the candidates’ stability by performing a pair-wise comparative analysis across HKGs. All 8 candidate HKGs showed strong correlation (0.69<r<0.93) and were combined into an index. Subsequently, the correlations between each HKG and the index were computed. The highest Pearson correlation coefficient (r) value for the relationship between the index and the contributing HKGs was obtained for RPLP0 (r = 0.93, P = 0.001) (Figure 2).

The geNorm applet calculates a gene expression stability M based on the geometric average V between all tested genes. All 8 studied genes had M values below the default limit of 1.5, which demonstrated that all genes tested had high expression stability. After stepwise exclusion of the worst-scoring HKGs, B2M and ACTB were identified as the two most stably expressed genes in the studied samples (Figure 3).

Finally, the NormFinder program was used to rank candidate HKG stability. This applet uses a model-based approach to estimate the intragroup and intergroup expression variation, and then combines them into a stability value that makes ranking the candidate genes across different disease status possible. The best gene identified by this program was B2M (Figure 4), and the best combination of two genes was B2M and RPLP0. Although the order of stability of the studied genes differed slightly among the 3 applets, the top 2 ranked genes exhibited similarly.

Expression Levels of Candidate HKGs in Three Groups of Subjects

Both B2M and RPLP0 were the top two most stable HKGs as generated by the three different analytical methods. Thus, a normalization factor (NF) based on the geometric mean of the best-performing HKGs was calculated by geNorm to compare the expression levels of the other candidate HKGs in the three subgroups. The results show that, before

**Table 1.** Demographic characteristics of the participants.

| Clinical characteristic       | Non-depressive asthmatics | Depressive asthmatics | Healthy controls |
|------------------------------|---------------------------|-----------------------|------------------|
| N                            | 10                        | 11                    | 10               |
| Sex, male:female             | 3:7                       | 2:9                   | 2:8              |
| Age (y)                      | 33.70±10.58               | 34.18±9.41            | 30.6±7.41        |
| BMI, kg/m²                   | 23.10±2.50                | 22.26±2.28            | 22.13±3.25       |
| Ex-smoker, n (%)             | 1 (10)                    | 0 (0)                 | 1 (10)           |
| Non-smoker, n (%)            | 9 (90)                    | 11 (100)              | 9 (90)           |
| Atopy, n (%)                 | 7 (70)                    | 4 (36.4)              | 4 (40)           |
| FEV1% predicted              | 91.12±15.69               | 82.28±17.07           | 116.86±15.21     |
| FVC% predicted               | 97.31±11.20               | 95.55±11.36           | 107.64±14.28     |
| FEV1/FVC%                    | 79.29±8.90                | 74.23±11.10           | 88.38±5.90       |
| PD20FEV1; Methacholine (mg)  | 0.50 (0.08–2.47)          | 0.94 (0.31–2.30)      | –                |
| ACT score                    | 16.10±4.84                | 14.91±2.70            | –                |
| Anaphylactic history, n (%)  | 8 (80)                    | 6 (54.5)              | 1 (10)           |
| GINA severity                |                           |                       |                  |
| Mild, n (%)                  | 1 (10)                    | 0 (0)                 | –                |
| Moderate, n (%)              | 5 (50)                    | 4 (36.4)              | –                |
| Severe, n (%)                | 4 (40)                    | 7 (63.6)              | –                |
| AQLOQ*                       | 4.45±0.87                 | 3.96±0.68             | –                |
| Eosinophils (10⁹/L)*         | 0.24 (0.06–0.57)          | 0.20 (0.06–1.22)      | 0.15 (0.02–0.49) |
| Eosinophils (%)              | 4.35 (1.10–10.5)          | 5.10 (1.6–14.1)       | 2.45 (0.3–11.6)  |
| Total IgE (IU/ml)*           | 190.88 (34.17–1002.29)    | 63.82 (0.05–547.81)   | 44.41 (8.11–189.05) |

Asthma medication used in past year (discontinued at least 2 weeks before recruitment)

|                          |                          |                      |                  |
|--------------------------|--------------------------|----------------------|------------------|
| ICS + LABA, n (%)        | 1 (10)                   | 2 (18.18)            | –                |
| Theophylline, n (%)      | 4 (40)                   | 3 (27.27)            | –                |
| Antileukotrienes, n (%)  | 1 (10)                   | 1 (9.09)             | –                |

*: mean ± SD;  
**: median (range);  
*P<0.05;  
**P=0.073; BMI, body mass index; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; ACT, Asthma control test; GINA, Global initiative for asthma; AQLOQ, Asthma quality of life questionnaire; Ig, immunoglobulin; PD20FEV1, provocative dose of methacholine causing a 20% drop in FEV1; ICS, inhaled corticosteroid; LABA, Long-acting β2-agonists.  
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normalization, the expression levels were not significantly different among the three groups for all candidate genes. After normalization, the expression levels of \( \text{PPIA} \) were significantly different between the three groups; Post hoc analysis by Student-Newman-Keuls test shows that depressive asthmatics and non-depressive controls \( (p<0.05) \) (Figure 5).

**Discussion**

The literature [6] overwhelmingly confirms that \( \text{CD4}^+ \) \( \text{T} \) cells play an important role in the occurrence and development of asthma, and an increasing amount of evidence [7,16] supports the concept that these cells also influence susceptibility to depression. The results of the present study will enable meaningful interpretation of data from expression studies that use purified \( \text{CD4}^+ \) \( \text{T} \) cells, and therefore can help understanding of underlying mechanisms.

Real-time quantitative PCR is a routinely used technique to measure transcript abundance with great sensitivity, specificity and reproducibility. Nevertheless, exact normalization of gene expression levels is an absolute prerequisite for reliable results of qPCR quantification methods.

This study demonstrates the use of three different Excel-based applets to identify the most stable HKGs in the studied population. Expression stability for a single sample or each HKG was investigated using BestKeeper first. All of the studied 28 samples had low InVar fold level. An InVar value of more than 3-fold expression levels is an absolute prerequisite for reliable results of qPCR. Nevertheless, exact normalization of gene expression levels is an absolute prerequisite for reliable results of qPCR quantification methods.

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The NormFinder uses a model-based approach to provide a more precise measure of gene expression stability due to its direct estimation of expression variation and consideration of systematic differences between subgroups, rather than pairwise comparison approach [14]. In addition, the pairwise comparison approach is probably influenced by HKG co-regulation, and therefore the final ranks may not be optimal.

\( \text{PPIA} \) encodes a member of the peptidyl-prolyl cis-trans isomerase (PPIase) family, which are ubiquitous intracellular proteins that play a role in cyclosporine A-mediated immunosuppression [17]. The role of \( \text{PPIA} \) in allergic asthma is inconsistent in the literature. On one hand, \( \text{PPI}^+/− \) knockout mice developed allergic disease accompanied by elevated \( \text{IgE} \) and an increased number of mast cells and eosinophils in multiple tissues, which was caused by type 2 cytokines released from \( \text{CD4}^+ \) \( \text{T} \) cells [18]. While on the other hand, increasing evidence has suggested that cyclophilins are potent chemoattractants for a variety of human and mouse leukocyte subsets [19,20]. Indeed, elevated protein levels of cyclophilin have been observed both in acute allergic asthma [21] and chronic periods of the disease. Blocking the function of \( \text{PPIA} \) reduced the recruitment of leukocytes and acute episodes of the disease following allergen challenge [22]. In the present study, \( \text{PPIA} \) mRNA level was lower in asthmatics than in healthy controls. One explanation is that in the present study,

| **Table 2.** Data of candidate housekeeping genes (n = 28). |
|-----------------------------------------------|
| **n** | **RN28S1** | **RPLP0** | **ACTB** | **PPIA** | **PGK1** | **B2M** | **GUSB** | **RPL13A** |
| GM 14.90 | 26.69 | 26.37 | 27.54 | 28.63 | 24.36 | 32.54 | 25.99 |
| AM 14.94 | 26.72 | 26.39 | 27.57 | 28.67 | 24.38 | 32.56 | 26.02 |
| min 12.83 | 23.77 | 23.83 | 23.93 | 24.33 | 22.33 | 30.07 | 24.00 |
| max 16.46 | 28.23 | 28.65 | 29.82 | 31.10 | 26.49 | 35.22 | 28.43 |
| SD 0.92 | 0.97 | 1.02 | 0.89 | 1.20 | 0.88 | 0.83 | 0.95 |
| CV (%) 6.19 | 3.65 | 3.85 | 3.22 | 4.19 | 3.63 | 2.56 | 3.66 |

GM, geometric mean; AM, arithmetic mean; Min, minimal value; Max, maximal value; CV, coefficient of variance.

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| **Table 3.** Housekeeping genes evaluated in the present study. |
|-----------------------------------------------|
| **Full name** | **Symbol** | **Gene function** | **Accession no.** |
| RN, 28S ribosomal 1 | RN28S1 | Ribosomal units | ENST00000419932 |
| Ribosomal protein, large, P0 | RPLP0 | Structural component of the 60S subunit of ribosomes | NM_001002.3 |
| Actin, beta | ACTB | Cytoskeletal structural actin | NM_001101 |
| Cyclophilin A | PPIA | Accelerate the folding of proteins | NM_021130.3 |
| Glyceraldehyde-3-phosphate dehydrogenase | GAPDH | Enzyme in glycolysis and nuclear functions | NM_002046 |
| Phosphoglycerate kinase 1 | PGK1 | Glycolytic enzyme | NM_00291.3 |
| Beta-2-microglobulin | B2M | Component of the major histocompatibility complex class I molecules | NM_004048.2 |
| Glucuronidase, beta | GUSB | Hydrolase that degrades glycosaminoglycans | NM_007181.3 |
| Ribosomal protein L13a | RPL13A | Structural component of the 60S ribosomal subunit | NM_012423.2 |

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unstimulated CD4+ T cells were studied. It is possible that PPIA level is low in resting CD4+ T cells. Upon allergen stimulation, such as in acute asthmatics or chronic asthmatics with continuous allergen exposure, PPIA expression would be higher than normal. This phenomenon was seen previously with other chemoattractants such as eotaxin, RANTES, MIP-1α, and MCP-1 [23,24,25]. Our previous study identified PPIA as a stable expressed HKG in airway epithelial cells [26], this paper has provided helpful information to a dozen of studies since its publication (citations from Google Scholar). Several publications used PPIA as a HKG to normalize the expression levels of target genes and found meaningful differential expressions of target genes [27,28]. Current study identified B2M and RPLP0 as the most optimal HKGs in gene expression studies involving human blood CD4+ T cells derived from normal subjects and asthmatics with and without depression. The different results from the two studies may be explained by the fact that the cell types in the two studies were different and our results have also strengthened the importance of optimal HKGs selection before performing any qRT-PCR in different disease conditions. Since asthma with depression have been considered to influence the disease process of asthma certainly, exploring the underlying pathophysiological mechanisms is necessary. However, before we determine the molecular basis, selecting optimal HKGs is the first and crucial step.

Conclusions

To our knowledge, this is the first study to identify the most stable HKGs in CD4+ T cells and depressive/non-depressive asthmatic disease status. B2M and RPLP0 were identified as the most optimal combination of HKGs in gene expression studies involving human blood CD4+ T cells. Furthermore, careful comparison of the gene expression profiles of purified CD4+ T cells based on information from this study will further elucidate the molecular basis of the incidence and development of asthma with or without depression.

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Figure 3. Ranking the housekeeping genes (HKGs) according to their expression stability $M$ determined using geNorm. A stepwise exclusion of the least stable HKG was conducted to obtain the mean expression stability value $M$ of remaining HKGs until the two most stable HKGs were identified. The genes are ranked according to $M$ values.

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Figure 4. Determination of the housekeeping gene expression stability by NormFinder. The stability value is estimated using the model-based approach. Having considered both the intra- and inter-group variation, a lower stability value represents a smaller systematic error that would be introduced when using the studied gene.

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Author Contributions

Conceived and designed the experiments: YLJ ZAL TW JQH. Performed the experiments: TW XYX YYY. Analyzed the data: TW. Contributed reagents/materials/analysis tools: TW XYX YYY. Wrote the paper: TW AJS JQH. Interpreted the results: TW AJS JQH.

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Figure 5. Comparison of the normalized relative expression levels of housekeeping genes (HKGs) between the three subgroups. The relative expression levels of remaining seven genes were normalized against the Normalization Factor based on the geometric mean of the expression level of the best-performing HKGs (B2M and RPLP0). Data are presented as mean ± SE. *P<0.05. doi:10.1371/journal.pone.0048367.g005

Selection of Suitable Housekeeping Genes
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