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Evaluation of Reference Genes for Studies of Gene Expression in Human Adipose Tissue

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Abstract

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Objective: The aim of this study was to evaluate reference genes for expression studies of human adipose tissue.

Research Methods and Procedures: Using 52 human adipose tissue expression profiles (HU95), 10 putative reference genes with the lowest variation in expression levels were selected for further studies. Expression stability of these 10 novel and 5 previously established reference genes was evaluated by real-time reverse transcriptase-polymerase chain reaction analysis. For this purpose, 44 adipose tissue biopsies from 27 subjects were chosen to include a wide range of parameters such as sex, age, BMI, depot origin, biopsy procedure, and effects of nutrition.

Results: *LRP10* was identified as the gene with the least variation in expression levels. The frequently used reference genes *RPLP0, 18S rRNA, PPIA, ACTB,* and *GAPD* were ranked as 4, 6, 7, 8, and 10, respectively.

Discussion: Our results suggest that *LRP10* is a better choice as reference for expression studies of human adipose tissue compared with the most frequently used reference genes.

Key words: housekeeping, bootstrapping, LRP10, β-actin, GAPD

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Introduction

A survey of 40 studies published since 2001 shows that, in 70% of the papers, *ACTB*, *GAPD*, and *18S rRNA* were used as reference genes for reverse transcriptase-polymerase chain reaction (RT-PCR)[1] measurements of gene expression in human adipose tissue or adipocytes. However, the expression of these genes has been reported to vary considerably in other tissues and cells (1–3). In addition, we have previously observed that *ACTB* was regulated during diet-induced weight loss (4). This study was, therefore, performed to identify and evaluate novel reference genes for analysis of gene expression in human adipose tissue and to compare these with frequently used reference genes.

Research Methods and Procedures

Subjects and Samples

This study was approved by the Medical Ethics Committee at Göteborg University, and all participants gave written informed consent. All biopsies were taken after an overnight fast, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis. Seventy-two biopsies from 27 subjects (6 men and 21 women) were used for the microarray expression profiling, and 44 biopsies from 27 subjects (14 men and 13 women) were used for the real-time RT-PCR analysis (supplemental data available online at http://www.obesityresearch.org). Procedures for RNA isolation and hybridization to the microarrays have been described previously (5,6).

Selection of Stably Expressed Genes in Human Adipose Tissue using Microarray Data

In total, 52 expression profiles of human adipose tissue from the above-mentioned 72 biopsies were used for the selection (supplemental data available online at http://www.obesityresearch.org). To perform a pre-selection using the largest group of data ($n = 36$), the 50 genes with the lowest

[1] Nonstandard abbreviations: RT-PCR, reverse transcriptase-polymerase chain reaction; CV, coefficient of variation.

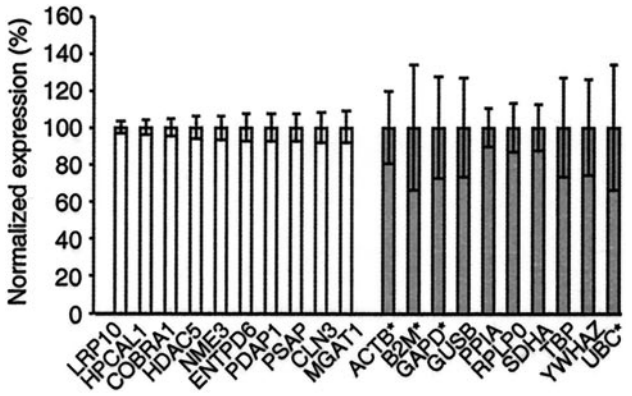

Figure 1: Expression stability of 10 novel (white bars) and 10 established (gray bars) reference genes in human adipose tissue analyzed by microarrays. The data are shown as normalized expression for each gene, and error bars indicate the CV, which was used to evaluate the expression stability of the genes (see Research Methods and Procedures).

coefficients of variation (CVs, %SD) were selected from expression profiles of paired subcutaneous and omental adipose tissue (5,6). Subsequently, all microarrays were used to create eight different groups representing different physiological variables of interest (supplemental data available online at http://www.obesityresearch.org). The average expression for each group was calculated, followed by computation of the CVs for the 50 genes across the eight groups. The 10 genes with the lowest CV were selected for subsequent studies (Figure 1; supplemental data available online at http://www.obesityresearch.org).

ACTB, *GAPD,* and *18S* were identified by PubMed searches as common reference genes for expression studies of human adipose tissue. Two other frequently used reference genes, *PPIA* and *RPLP0*, with low CV in the microarray data, were also included (Figure 1). The 5 established and 10 novel putative reference genes were evaluated by real-time RT-PCR analysis on individual samples.

Real-time RT-PCR

Eleven of the 15 genes were analyzed using predesigned TaqMan Assays-on-Demand (Applied Biosystems, Foster City, CA). Probe-primer sets for the four remaining genes, *COBRA1, ENTPD6, PDAP1,* and *HDAC5,* were designed using the Primer Express software v2.0 (Applied Biosystems). TaqMan Reverse Transcriptase reagents, TaqMan Universal PCR Master mix (Applied Biosystems), and reaction conditions were used according to the manufacturer's instructions (supplemental data available online at http://www.obesityresearch.org).

Ranking the Putative Reference Genes

Ranking of the selected reference genes was performed essentially as described by Vandesompele et al. (7). Briefly, for each gene, the gene expression ratio versus all other genes was calculated in each sample. Subsequently, for each pair of genes, a pairwise variation was defined as the SD of the pairwise log ratios for all samples, and for each gene, a gene instability measure was defined as the mean overall of the pairwise variations for that gene.

An iterative process was employed in the ranking procedure where genes were excluded in a stepwise manner. In each step, the gene with the highest gene instability measure was excluded, after which new gene instability measures were calculated using only the remaining genes. This procedure was repeated until only three genes remained. These genes were ranked as first, second, and third according to their gene instability measures.

In addition, we performed a bootstrap step to evaluate the certainty of the ranking. The ranking method was bootstrapped (8) by resampling with replacement from the original set of 44 sample files. The resampling procedure was repeated 10,000 times (supplemental data available online at http://www.obesityresearch.org). To check the robustness of the ranking procedure with respect to outliers, we also repeated the ranking with trimmed SDs, excluding the most outlying 10%, 20%, and 40% of log ratios in the computation of the SDs of the pairwise log ratios.

Results

Selection of Putative Reference Genes in Human Adipose Tissue from Microarray Data

Based on the analysis of data from 52 microarrays, the 10 genes with the least variability in expression levels were selected as possible novel reference genes. Figure 1 shows the normalized signal and the CVs for each of the 10 genes together with established reference genes represented on the same microarrays.

Evaluation of Gene Expression Stability

To evaluate the expression stability of the selected genes, we collected samples from both sexes with wide variation with respect to age (20 to 64 years), BMI (20.5 to 51.2 kg/m^2), nutrition (before diet, 8 weeks of diet, and 2 weeks refeeding after completed 16-week diet), depot (omental, subcutaneous), biopsy procedure (surgical, needle), and anesthesia (local, general). In total, 44 adipose tissue biopsies from 27 subjects were used for gene expression analysis by real-time RT-PCR. The assays for *PDAP1* and *MGAT1* yielded no or very low signals; consequently, only 13 genes were analyzed further.

The results from the ranking of the genes and the evaluation of the certainty of ranking by bootstrap procedure are shown in Figure 2. The figure shows that *LRP10* was ranked first in 71% of 10,000 bootstrap samples, followed by *CLN3*, ranked first in 24% and second in 51% of the bootstrap samples. These results obtained by the bootstrap

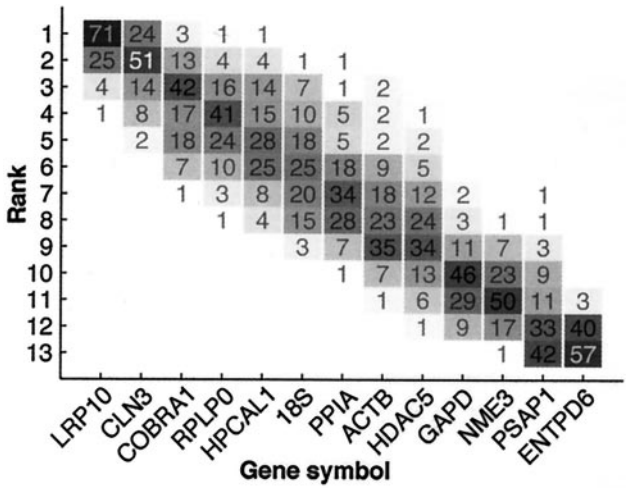

Figure 2: Empirical distribution of ranks shown in percentage for the 13 remaining genes analyzed by real-time RT-PCR in bootstrap of size 10,000. Stable genes have low ranks. The varying shading of numbers is only for improved readability of the figure.

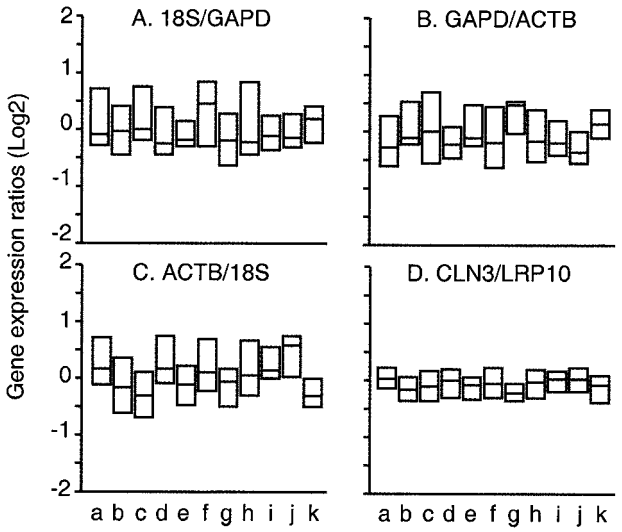

Figure 3: Variation in expression ratios of different gene combinations in human adipose tissue analyzed by real-time RT-PCR. (A) *18S/ACTB*. (B) *GAPD/ACTB*. (C) *ACTB/18S*. (D) *CLN3/LRP10*. The panels show box plots of the gene expression ratios in adipose tissue from (a) women ($n = 12$), (b) men ($n = 13$), (c) subjects with BMI <30 kg/m^2 ($n = 12$), (d) subjects with BMI ≥ 35 kg/m^2 ($n = 13$), (e) age <40 years ($n = 13$), (f) age ≥ 40 years ($n = 12$), (g) omental depot ($n = 9$), (h) subcutaneous depot ($n = 7$), (i) obese subjects, before weight loss ($n = 8$), (j) obese subjects, during weight loss ($n = 8$) and (k) obese subjects, after weight-loss ($n = 4$). The box plots show the quartiles of gene expression ratios where the expression of each gene was normalized before calculation of the ratios.

procedure were in agreement with the original ranking. Furthermore, the ranking was also robust in that it was essentially unaffected by trimming away 10%, 20%, or 40% of the most outlying log ratios. The results suggest that *LRP10* is the most stably expressed gene.

Effects of Sex, BMI, Age, Depot, and Biopsy Procedure

To evaluate how the choice of reference gene affects the final results, the analyzed samples were grouped according to sex, BMI, age, depot origin, and effect of diet. Given that *18S*, *GAPD,* and *ACTB* are frequently used as reference genes, we chose to show the variation that was introduced when *18S* expression was related to *GAPD*, *GAPD* expression was related to *ACTB,* and *ACTB* expression was related to *18S* (Figure 3, A–C). The expression ratios of the two top ranked genes, *CLN3* and *LRP10*, are shown for comparison (Figure 3D). Figure 3 shows that the combination of *CLN3* and *LRP10* reduced the within-group variation substantially compared with the expression ratios of the other combinations of reference genes. Furthermore, *CLN3* and *LRP10* showed lower variation among the medians in the different groups, suggesting that the different conditions had very small effects on the expression of these genes. The full figure showing all possible combinations of reference genes is shown in supplemental data (data available online at http://www.obesityresearch.org).

Discussion

In this study, we used microarray data, real-time RT-PCR, and a bootstrap procedure to identify genes with low variation in expression levels in human adipose tissue. *LRP10* was identified as the gene with the highest expression stability in human adipose tissue biopsies that were selected to represent a wide range of commonly studied physiological parameters.

Several approaches have been made to adjust for sample variation in quantitative RT-PCR analysis. For example, gene expression has been related to total RNA or cell count (9). However, this approach lacks control of the cDNA synthesis step, and cell counts can be applied to studies performed only on cells and not tissues. Other strategies, such as spiking exogenous in vitro synthesized RNA (10,11), are time-consuming, which is why the majority of published studies use an endogenous reference gene. However, the evaluation of an optimal reference gene based on the real-time RT-PCR data becomes a circular problem because of the lack of an absolute reference point, as discussed by Vandesompele et al. (7) in their paper presenting a strategy for identifying the most stably expressed reference genes (7). The use of the bootstrap technique, which we have applied in this study, extends the method of Vandesompele et al. by enabling us to differentiate the expression stability of the two highest ranked genes.

ACTB, 18S, and *GAPD* are the most frequently used reference genes for expression studies of human adipose

tissue, but other reference genes have also been used (e.g., *PPIA* and *RPLP0*). We found that of the established reference genes, *RPLP0* was highest ranked. However, expression of *RPLP0* when related to *LRP10* was significantly affected by diet-induced weight loss. *GAPD* expression in adipose tissue generally showed a higher variation in all groups compared with *18S* and *ACTB*, which is in contrast to in vitro cultured human adipocytes, where *GAPD* is stably expressed (12).

Little is known of the biological function of *LRP10*. The mouse homologue to *LRP10*, *Lrp10,* also known as *Lrp9*, mediates cellular uptake and hydrolysis of cholesterol esters in apolipoprotein E-enriched very-low-density lipoproteins in vitro (13). In conclusion, based on the results of this study, we recommend the use of *LRP10* as a reference gene for expression studies of human adipose tissue.

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