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Evaluation of the reference genes in human adipose tissue and lipoma samples

Original Article

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Abstract:

Housekeeping genes, by definition and function, should be constitutively and stably expressed in all cells of the organism, regardless of the cell type and function they accomplish. However, it was observed that expression of housekeeping genes, used as reference genes in gene expression analysis, varies greatly in different cells and tissue types and is also dependent on the experimental conditions and treatments. The aim of our study was to examine the expression patterns of the most frequently used reference genes (*GAPDH*, *ACTB* and *RRN18S*) in the samples of human subcutaneous adipose tissue (scWAT) and benign adipose tissue tumors (lipomas). The obtained results have shown that the expression of all three examined housekeeping genes is slightly lower in lipoma samples compared to scWAT samples and that *GAPDH* is the most stable housekeeping gene in examined samples so it can be recommended as an optimal reference gene for gene expression analysis in human scWAT and lipoma samples.

Key words:

reference genes, gene expression, adipose tissue, lipoma, scWAT

Apstract:

Evaluacija referentnih gena u uzorcima humanog masnog tkiva i lipoma

Housekeeping geni bi, po definiciji i funkciji, trebalo da se konstitutivno i stabilno eksprimiraju u svim ćelijama organizma, nezavisno od tipa ćelija i funkcije koju one obavljaju. Primećeno je međutim da ekspresija *housekeeping* gena, koji se koriste kao referentni geni u analizi ekspresije gena, varira u velikoj meri zavisno od tipa ćelija i tkiva i da zavisi takođe od eksperimentalnih uslova i tretmana. Cilj našeg istraživanja je bio ispitati obrazac ekspresije najčešće korišćenih referentnih gena (*GAPDH*, *ACTB* i *RRN18S*) u uzorcima humanog potkožnog masnog tkiva (scWAT) i benignih tumora masnog tkiva (lipoma). Dobijeni rezultati su pokazali da je ekspresija sva tri ispitivana gena nešto niža u uzorcima lipoma u odnosu na uzorke scWAT, da je *GAPDH* najstabilniji *housekeeping* gen u ispitivanim uzorcima i da se može preporučiti kao optimalan referentni gen za analizu ekspresije gena u uzorcima humanog scWAT i lipoma.

Ključne reči:

referentni geni, genska ekspresija, masno tkivo, lipomi, scWAT

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Introduction

Housekeeping genes, by definition and function, should be constitutively and stably expressed in all cells of the organism, regardless of the cell type function and they accomplish, and are considered to be the minimal set of genes necessary for the life of an organism (Eisenberg and Levanon, 2013). Since they are essential for maintaining the basic cellular structures and functions, they are expressed at all stages of the development and growth of an organism, as well as in physiological and pathophysiological states (Eisenberg and Levanon, 2013). In biomedical research, housekeeping genes are widely used as reference genes, i.e. internal controls. The largest application in this field is for the purposes of gene expression analysis using qRT-PCR (quantitative reverse-transcription polymerase chain reaction) method, where housekeeping genes play a role of endogenous control for normalizing the expres-

sion of a gene of interest (GOI) (Bustin and Nolan, 2004; Eisenberg and Levanon, 2013; Caracausi et al., 2017). Many studies have shown that the results obtained using this method are largely influenced by various factors, such as experimental conditions, sample size and RNA isolation techniques, but also RNA integrity, and the efficiency of reverse transcription reaction (RNA reversely transcribed into cDNA) and the quality of the cDNA obtained (Bustin, 2002; Bustin and Nolan, 2004). The influence of these factors is due to the multistage manipulations of the samples prior to the qPCR reaction itself (RNA isolation, reverse transcription etc.), as well as the use of different reagents as obligatory components of the kits that are necessary during all the above steps. All of these can significantly affect the efficacy and specificity of the qPCR reaction itself, so certain internal controls need to be included in the analysis to increase efficiency and to make the final result as realistic and highly specific as possible for the studied GOI. Housekeeping (reference) genes are used to correct, i.e. to normalize variations in the expression of the GOI from sample to sample, thereby avoiding errors and increasing the efficiency of the RT-PCR method when quantifying the expression of the GOI.

In order for a housekeeping gene to be considered as the most suitable reference gene used for normalization in gene expression analysis it should meet the following criteria: to be expressed at a sufficient level so it can be easily detected, to have stable/constant expression in different cell types in an examined organism and within the same cell type undergoing different treatments or experimental conditions and to be ubiquitously expressed (Caracausi et al., 2017). Thus, the proper selection of the reference gene for studying gene expression by qRT-PCR is extremely important and can be very challenging (Andersen et al., 2004; Derveaux et al., 2010; Zhang et al., 2016). Although many different reference genes have been used in the research to date, no "ideal" reference gene has been found yet (Radonić et al., 2004; Andersen et al., 2004). The stability of the commonly used reference genes has been shown to be relative, since it was observed that their expression varies greatly in different cell and tissue types and also depending on the experimental conditions and different treatments (Thellin et al., 1999; Schmittgen and Zakrajsek, 2000; Suzuki et al., 2000; Warrington et al., 2000; Tricarico et al., 2002; Andersen et al., 2004; Dheda et al., 2004).

In the literature, among the reference genes used in the studies of gene expression in biomedical research, various genes can be found (Eisenberg and Levanon, 2013; Taube et al., 2015; Zhang et al., 2016; Almeida-Oliveira et al., 2017), however among the most frequently used, regardless of the sample type and organism, are genes for: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta actin (*ACTB*) and 18S ribosomal RNA (*RRN18S*) (Gabrielsson et al., 2005; Almeida-Oliveira et al., 2017), while in the studies that analyzed gene expression in different adipose tissue samples, in addition to these three the most common, genes for LDL receptor related protein 10 (*LRP10*), beta-2-microglobulin (*B2M*) and beta-glucuronidase (*GUSB*) were also used.

Lipomas represent one of the most common soft tissue neoplasms of mesenchymal origin (Mohammed et al., 2014; Omonte et al., 2016) with still unclear etiology and pathogenesis. Although lipomas are very common soft tissue tumors, there is insufficient information on their molecular characteristics and the potential mechanisms of their formation. To understand the mechanisms of lipoma formation and to find potential ways for their adequate treatment, detailed characterization of these tumors at molecular level needs to be performed in addition to already performed studies on the implications of mesenchymal stem cells in the pathogenesis of lipoma (Stojanović et al., 2018; Stojanović and Najman, 2019). Gene expression analysis gives us insight into the changes that have occurred in adipose tissue that may have led to its dysfunction and tumor development, so it is necessary to have reliable reference genes to perceive the real state of transcriptome and to find target places for eventual therapy. Considering the very different data on reference genes used in gene expression analysis and the extent to which their expression varies, both in different tissue types and in different samples of the same tissue type, the aim of this study was to examine the expression patterns of the most frequently used reference genes (*GAPDH*, *ACTB* and *RRN18S*) in the samples of human subcutaneous adipose tissue (scWAT) and benign adipose tissue tumors (lipomas).

Materials and methods

Tissue samples

Lipoma tissue samples were obtained after surgical removal of the lipomas, previously clinically diagnosed as benign adipose tissue tumors, while scWAT samples were taken during other non-cancerous surgeries patients underwent. All samples were obtained at Clinical Center Niš, Serbia, and all patients included in the study gave their informed written consent. The study was approved by the Local Ethics Committee of the Faculty of Medicine, University of Niš, Serbia (approvals 01-6481-15 and 12-6316-2/4). A total of 37 tissue samples were analyzed, of which 24 lipoma and 13 scWAT samples, patients of both sexes aged 21 to 70 years. Tissues

were sampled from several body localizations such as: head, oral cavity, neck, arm, leg, abdomen, back, hip, knee and groin. All patients had a body mass index less than 30, which excluded pathologically obese patients.

Gene expression analysis

RNA isolation and reverse transcription

Immediately after sampling, parts of the lipoma and scWAT were stored in RNAlater® at -80 °C until RNA isolation. Total RNA was isolated using RNeasy Lipid Tissue Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions, after tissue homogenization in TRIzol reagent (Qiagen), using tissue homogenizer (TH220, Omni international, SAD). On column removal of residual genomic DNA was performed during RNA isolation using DNase I RNase-free kit (Qiagen). RNA concentration was determined immediately after isolation on the Qubit® fluorimeter (Thermo Scientific, Waltham, MA, USA) using Qubit™ RNA BR Assay Kit (Thermo Scientific), and then isolated RNA was stored at -80 °C until further use. Total RNA was subsequently transcribed into a single-stranded cDNA using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The reverse transcription reaction was carried out in SureCycler8800 (Agilent Technologies, Santa Clara, CA, USA), with 400 ng of total RNA per reaction per sample. The reverse transcription reaction was performed according to the following program: 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C and cooling at 4 °C. The synthesized cDNA was stored at -80 °C and used later to determine relative gene expression.

Real-Time PCR

Analysis of the relative gene expression in tissue samples, by SYBR green based Real-Time qPCR method, was performed in Mic qPCR cycler (Bio Molecular Systems, Australia). The reactions were prepared using Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Universal ROX dye was used as a reference dye. Predesigned primer sets (QuantiTect primer assay kits, Qiagen) were used for the following genes: *GAPDH* (QT00079247), *RRN18S* (QT00199367) and *ACTB* (QT00095431). The protocol conditions were as follows: (1) enzyme activation: 60 s at 95 °C (1 cycle); (2) denaturation: 15 s at 95 °C and annealing/extension (with data acquisition): 30 s at 60 °C (40 cycles). The specific binding of primers was confirmed by melting curve analysis for each reaction and by visualizing specific length products on electrophoresis gel. The expression pattern of the examined housekeeping (reference) genes, as well as the variation of their expression among the samples of both study groups, was analyzed based on the obtained Ct (cycle threshold) values.

Statistical analysis

Results in the form of obtained Ct values were statistically processed. As indicators of central tendency mean and median values were calculated, while standard deviation (SD) and coefficient of variation (CV) were calculated as indicators of the variability. The results are presented as boxplots, made in GraphPad Prism 5, with median values and the range of minimum and maximum values. Statistically significant differences in the expression of the examined reference genes between lipoma and scWAT samples were analyzed by ANOVA and Mann – Whitney U tests. Values of $p < 0.05$ were considered as statistically significant.

Results

The specificity of qPCR reactions was confirmed by melting curve analysis and the results are presented in **Fig. 1**. Based on the obtained melting curves, it can be seen that in all qPCR reactions the specific primer binding products were obtained. Only one peak for *RRN18S* (a), *ACTB* (b) and *GAPDH* (c) for each qPCR sample reaction that can be observed in the graphs indicates the presence of only one specific reaction product per sample.

The results of the gene expression pattern analysis of *GAPDH*, *ACTB* and *RRN18S* housekeeping genes are presented as Ct values in **Fig. 2**. It can be noticed that *RRN18S* is highly expressed in all samples, as shown by low Ct values (**Fig. 2a**). The expression of *RRN18S* is slightly lower in lipoma than in scWAT samples, although the resulting difference is not statistically significant ($p = 0.86$). The expression of *RRN18S* is highly variable in both examined groups of samples, which can be noticed based on the distribution of Ct values (**Fig. 2a**). The expression of *ACTB* was statistically significantly lower in lipoma compared to scWAT samples ($p < 0.05$) (**Fig 2b**). Broad range and uneven distribution of the values can be noticed in both lipoma and scWAT group, indicating a relatively large variability in *ACTB* expression in different samples within both study groups (**Fig. 2b**). The results of *GAPDH* expression (**Fig. 2c**) indicate statistically significantly lower level of expression of this gene in lipoma compared to scWAT samples ($p < 0.05$), which can be seen from the higher Ct values in lipoma samples. On the other hand, narrower range and more uniform distribution of Ct values can be observed in the case of *GAPDH* expression compared to the expression

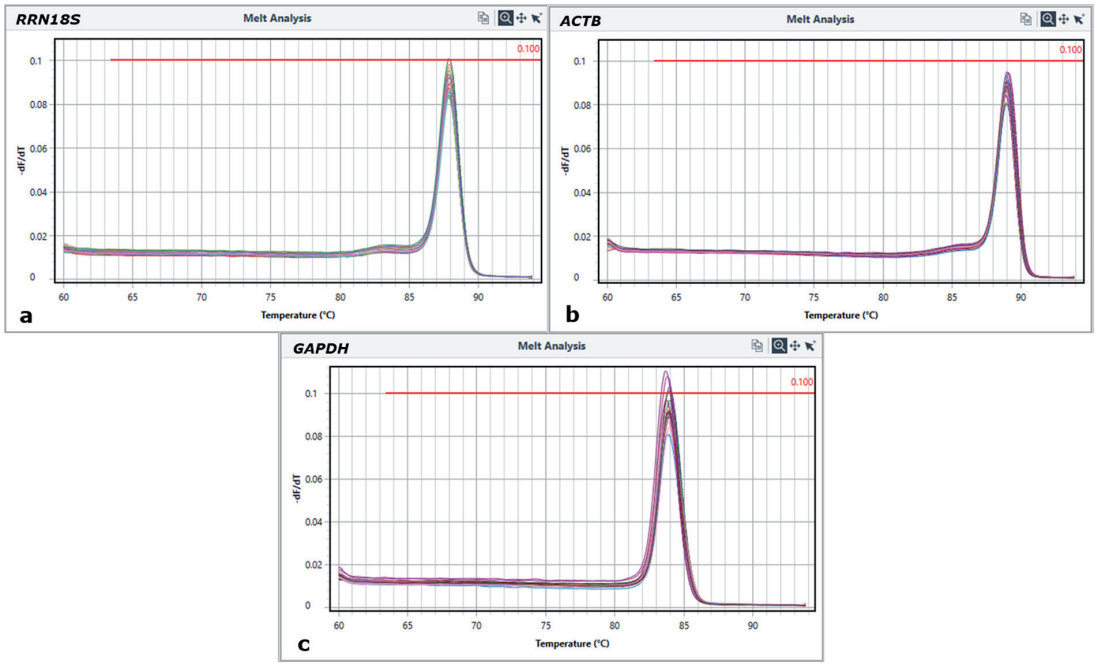

Fig. 1. Melting curves for all qPCR products; reference genes: *RRN18S* (a), *ACTB* (b) and *GAPDH* (c)

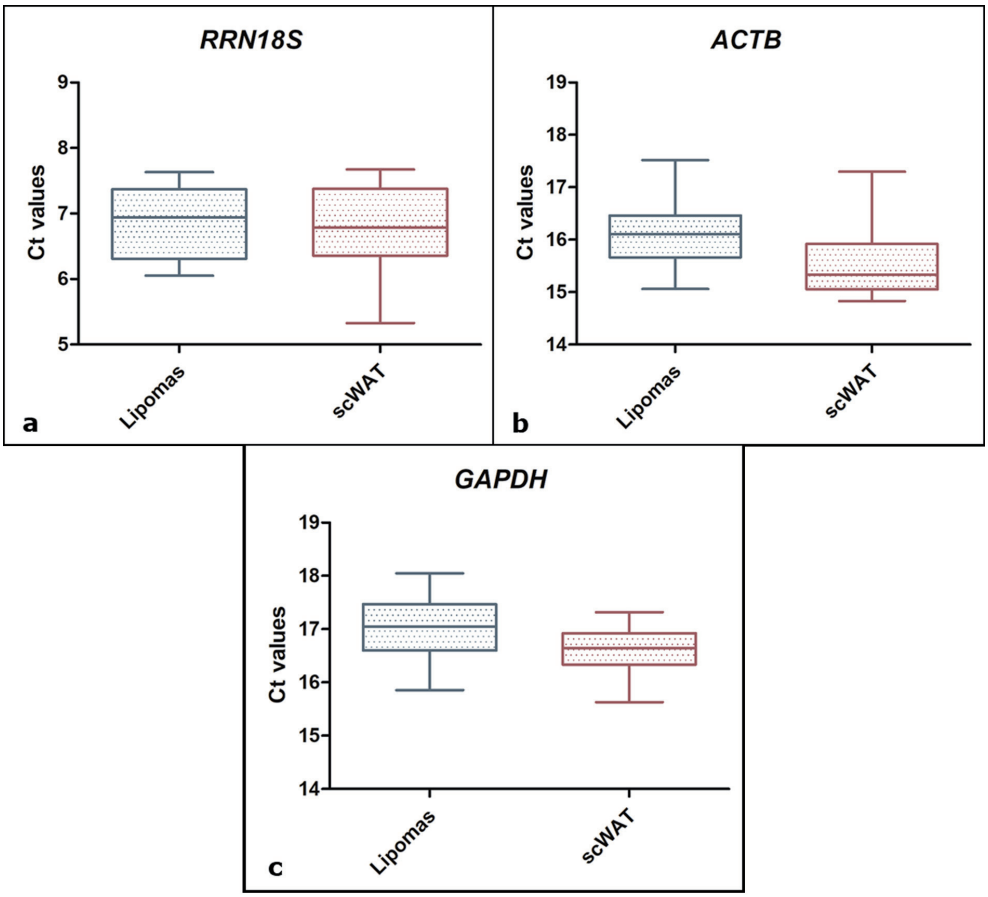

Fig. 2. Expression pattern of *RRN18S* (a), *ACTB* (b) and *GAPDH* (c) housekeeping genes in lipoma and scWAT samples; the results are presented as Ct values; boxplot charts with median and the range of minimum and maximum values

of previously analyzed *RRN18S* and *ACTB* genes.

The CV, as a measure of deviation from the mean, is the lowest in the case of *GAPDH* gene in both lipoma and scWAT group of samples (**Tab. 1**) which confirms the findings that *GAPDH* is the most stable reference gene in our study in lipoma and scWAT samples. For all tested genes, mean and median Ct values were higher in lipoma compared to scWAT group of samples, indicating that the expression of all three examined reference genes was slightly lower in lipoma samples compared to scWAT samples. Statistically significant difference ($p < 0.05$) in gene expression between lipoma and scWAT samples was noticed for *ACTB* and *GAPDH*, but not for *RRN18S*. Similar values of the mean and median can be observed for all tested samples in the case of all examined genes, indicating a relatively symmetric distribution of values in both groups.

Discussion

To adequately analyze the expression of GOI in examined samples and to increase the efficiency of qPCR reactions, it is very important to choose the optimal housekeeping gene. *GAPDH*, *ACTB* and *RRN18S* are the most commonly used reference genes that can be found in published studies that dealt with gene expression analyses wherein *GAPDH* was recommended as the reference gene with the most stable expression. However, numerous literature data indicates that there is a great variability in the expression of these genes both in samples of different tissues and samples of the same tissue and cell type as well as under different experimental conditions, treatments and metabolic states. There are various computer programs, statistical methods and software tools such as NormFinder, geNorm, BestKeeper and Global Pattern Recognition for identification and selection of an optimal reference gene (De Spiegelaere et al., 2015; Perez et al., 2017) that can predict the expression pattern of housekeeping genes in the desired samples, but they cannot confidently predict the experimental conditions and how it will affect the expression of reference genes. When adipose tissue is analyzed good prediction of this type cannot be done since adipose tissue is an endocrine and paracrine organ that is greatly influenced by various factors such as nutrition, hormonal status, metabolic status, physical activity, temperature, etc. and also its characteristics vary from organism to organism and within different anatomical localizations of the same organism. Therefore, the aim of this study was to examine the expression pattern of three the most commonly used housekeeping genes in the scWAT and lipoma samples, and to determine which housekeeping gene is the most optimal to be used as a reference gene in gene expression analysis by real-time PCR. Due to inter- and intraindividual differences in housekeeping gene expression in human tissue samples, some authors consider that, in the case of comparison, more than one housekeeping gene should be used to normalize the level of the RNA of the target gene (Tricarico et al., 2002).

In the study that analyzed the expression patterns of housekeeping genes in colon and bladder cancer samples by RT-PCR, housekeeping genes *UBC*, *GAPDH* and *TPT1* were shown to be suitable for normalization in colon cancer samples, while *HSPCB*, *TEGT* and *ATP5B* were suitable for normalization in bladder cancer samples (Andersen et al., 2004). In the study in which the expression of several housekeeping genes was analyzed in 16 different human tissue samples by real-time PCR, the *RPII* gene has been shown to be suitable as a reference gene for a large number of different tissues (Radonić et al., 2004), but authors have noticed that the expression level of examined genes was signifi-

Table 1. Statistical parameters of reference genes' expression analysis in lipoma and scWAT samples

	RRN18S		***ACTB***		***GAPDH***	
	Lipomas	scWAT	Lipomas	scWAT	Lipomas	scWAT
Mean	6.89	6.82	16.13	15.58	17.03	16.61
Median	6.94	6.79	16.11	15.33	17.05	16.65
Minimum	6.05	5.33	15.06	14.83	15.86	15.63
Maximum	7.63	7.67	17.52	17.30	18.05	17.32
Standard deviation	0.53	0.68	0.64	0.79	0.58	0.44
Coefficient of variation (%)	7.69	9.97	3.97	5.10	3.41	2.65
Statistical significance	$p = 0.86$		$p < 0.05$		$p < 0.05$	

cantly different in different tissue types. In the same study, in performed *in vitro* experiments on cell cultures, it has been shown that different treatments have the least effect on *RPII* expression (Radonić et al., 2004). When expression level of 10 commonly used reference genes was analyzed (*GADPH*, *PPIA*, *ACTB*, *YWHAZ*, *RRN18S*, *B2M*, *UBC*, *TBP*, *RPLP* and *HPRT*) in human heart tissue samples, *PPIA*, *RPLP* and *GADPH* genes have been shown to be the most stable as estimated by geNorm and NormFinder and were recommended for the analysis of this tissue type (Pérez et al., 2007). No statistically significant difference was found between the rats on the lipid-rich diet and the control group when the expression levels of the reference genes *Rrn18S*, *Actb*, *Gapdh* and *36B4* were examined in three different types of rat adipose tissue of both groups of rats, but it was noticed that *Rrn18S* was the most expressed gene in all samples (Zhang et al., 2016) which correlates with the results obtained in our study. The stability of reference genes' expression was examined by GeNorm, NormFinder and BestKeeper softwares and the results showed that *Gapdh* was the most stable reference gene in eWAT (epididymal white adipose tissue) samples, but in iBeAT (inguinal beige adipose tissue) and BAT (brown adipose tissue) samples its expression was the most variable (Zhang et al., 2016). The results obtained with geNorm and NormFinder differed from those obtained by other analyzes, so the authors proposed *36B4* and *Gapdh* as the best combination of reference genes for eWAT samples, while for iBeAT and BAT samples they suggested *36B4* and *Actb* (Zhang et al., 2016). Changes in the expression of housekeeping genes were monitored during adipogenic differentiation of primary preadipocytes as well as under various experimental conditions and treatments of human primary mature adipocytes and its has been shown that the expression of *GAPDH* and *TfR* housekeeping genes was the most stable in different experimental conditions (Gorzelniak et al., 2001). The effect of adipogenic differentiation, cryopreservation and medium supplementation on the stability of 11 reference genes was examined in ADSCs *in vitro* and estimated by GeNorm software (Dessels and Pepper, 2019). It has been shown in this study that different experimental conditions significantly influence the expression stability of reference genes while the most stable expression in all experimental conditions was observed for *YWHAZ*, *HPRT*, *TBP* and *ACTB* (Dessels and Pepper, 2019). Expression of 12 commonly used reference genes was analyzed in different murine adipose tissue samples of animals subjected to different treatments and the results showed that the *Tbp* gene has stable expression in examined adipose tissue samples of the control group and is proved to be suitable for the analysis of gene expression in BAT of the control and obese mice (Almeida-Oliveira et al., 2017). The *Atpf1* gene was stably expressed in the subcutaneous and perigonadal adipose tissue samples of control and obese mice and was proved to be the best for normalizing gene expression in adipose tissue samples of streptozotocin-treated animals (Almeida-Oliveira et al., 2017). Authors of this study conclude that there is no ideally stable reference gene under all experimental conditions, but on the basis of the results obtained, they propose *Tbp* and *Atpf1* as suitable reference genes for gene expression normalization in mouse adipose tissue samples in various metabolic states and disorders (Almeida-Oliveira et al., 2017). When several commonly used reference genes as well as some new candidates were analyzed by qPCR and microchip analysis in perirenal BAT and subcutaneous adipose tissue, among commonly used, *RPLP0*, *LRP10*, *YWHAZ* and *GAPDH* showed the lowest variability in examined samples in this study as evaluated by NormFinder and geNorm software (Taube et al., 2015). The authors of this study conclude that all frequently used reference genes, except *RRN18S*, show acceptably low variability (Taube et al., 2015). Also, authors identified potentially new reference genes such as: *GNB1*, *GNB2* and *PSMB2*, which proved to be even more stable than the commonly used reference genes (Taube et al., 2015). Our results have shown that the expression of *GAPDH* was the most stable in both groups of examined samples (lipoma and scWAT), and varied the least between different patients within examined groups.

Expression analysis of a large number of reference genes in subcutaneous and omental adipose tissue samples has shown that *RPLP0* is the most suitable reference gene for adipose tissue. However, in one study it was shown that the expression of *RPLP0*, when analyzed in relation to the *LRP10*, was significantly influenced by the diet-induced weight loss (Gabrielsson et al., 2005). In examined adipose tissue samples in that study, it was shown that *GAPDH* expression varied in greater extent in all examined groups compared to *RRN18S* and *ACTB*, which is contrary to what was observed in cultures of primary human adipocytes *in vitro* for the same genes, where expression of *GAPDH* was stable in all samples and experimentally conditions (Gorzelniak et al., 2001). This finding in tissue samples is contrary to what we obtained in our study. Gabrielsson et al. (2005) propose *LRP10*, as the most suitable reference gene for gene expression analysis in human adipose tissue samples.

Expression of *IPO8* and *FBXL10*, as potential reference genes, was examined by real-time PCR in omental and subcutaneous adipose tissue samples as

well as in cultured primary preadipocytes and it has been shown that *IPO8* expression was more stable in tissue samples of both adipose tissue depots as well as in preadipocytes isolated from subcutaneous adipose tissue, compared to *FBXL10* expression that was more stable in preadipocytes from omental adipose tissue (Hurtado del Pozo et al., 2010). As the expression level of these two genes did not change during adipogenesis, the authors of this study concluded that both *IPO8* and *FBXL10* are good candidates for reference genes for gene expression analysis in human omental and subcutaneous adipose tissue samples as well as in differentiation of preadipocytes isolated from these tissues (Hurtado del Pozo et al., 2010). Perez et al. (2017) analyzed the expression stability of a large number of reference genes by ΔCt method in GeNorm, NormFinder and BestKeeper software, using different adipocyte and muscle cell cultures *in vitro*, under different treatments and conditions, in animal muscle and adipose tissue samples of different metabolic states as well as in human muscle and adipose tissue samples. Different results were obtained for different tissue types of different origin and under different conditions but general observation was that *RRN18S* was highly expressed in human samples and that gene for β-actin, although often used alone as a reference gene in RT-qPCR, is one of the most unstable genes in all muscle and adipose tissue samples, and is not recommended for this type of research (Perez et al., 2017).

The selection of an optimal reference gene for gene expression analysis is of great importance, because its stability in different samples and conditions will determine the final results obtained for the expression of GOI and their reliability. Zhang et al. (2016) demonstrated how the reference genes influence the expression of *Lep* and *Ucp-1* gene in different adipose tissue samples of rats on a high-fat diet and control normal-fed rats. The expression of both examined GOI was normalized by the expression of the most stable and the least stable reference genes. Different gene expression patterns were obtained for *Lep* and *Ucp*-1 gene in different adipose tissue samples and different groups of animals when expression was normalized with the most and the least stable reference genes previously determined for each type of examined adipose tissue (Zhang et al., 2016).

Based on the literature data, we can conclude that it is difficult to find an "ideal" reference gene that will have stable expression in different tissue types and samples of the same tissue type but under different experimental conditions and treatments. It is a common occurrence that one reference gene is suitable for one tissue type or treatment but not for the others, so it is difficult to compare the expression of GOI between different samples. In our study, *GAPDH* was shown to be the least variable and the most stable examined housekeeping gene in both lipoma and scWAT group which is of great importance for the gene expression analysis and comparison between lipoma and scWAT samples that could reveal the molecular mechanisms involved in the formation of lipoma. It can be noted that the expression of all examined housekeeping genes is slightly lower in lipoma samples compared to scWAT, which obviously represents a characteristic of lipomas and reflects their basal metabolic status.

Conclusion

Based on the obtained results we can conclude that the expression of all three examined housekeeping genes (*RRN18S*, *ACTB* and *GAPDH*) is slightly lower in lipoma samples compared to scWAT samples and that expression of *GAPDH* is the least variable and the most uniform in both examined groups of samples. We can consider *GAPDH* as the most stable housekeeping gene in our study and recommend it as an optimal reference gene for gene expression analysis in human scWAT and lipoma samples and its use for normalization of gene expression in real-time PCR method.

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References

Almeida-Oliveira, F., Leandro, J.G.B., Ausina, P., Sola-Penna, M., Majerowicz, D. 2017: Reference genes for quantitative PCR in the adipose tissue of mice with metabolic disease. *Biomedicine & Pharmacotherapy*, 88: 948-955.

Andersen, C.L., Jensen, J.L., Ørntoft, T.F. 2004: 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 Research*, 64(15): 5245-50.

Bustin, S.A. 2002: Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology*, 29(1): 23-39.

Bustin, S.A., Nolan, T. 2004: Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *Journal of Biomolecular Techniques*, 15(3): 155-66.

Caracausi, M., Piovesan, A., Antonaros, F., Strippoli, P., Vitale, L., Pelleri, M.C. 2017:

Systematic identification of human housekeeping genes possibly useful as references in gene expression studies. *Molecular Medicine Reports*, 16(3): 2397–2410.

Derveaux, S., Vandesompele, J., Hellemans, J. 2010: How to do successful gene expression analysis using real-time PCR. *Methods*, 50(4): 227-30.

De Spiegelaere, W., Dern-Wieloch, J., Weigel, R., Schumacher, V., Schorle, H., Nettersheim, D., Bergmann, M., Brehm, R., Kliesch, S., Vandekerckhove, L., Fink, C. 2015: Reference gene validation for RT-qPCR, a note on different available software packages. *PLoS One*, 10(3): e0122515.

Dessels, C., Pepper, M.S. 2019: Reference Gene Expression in Adipose-Derived Stromal Cells Undergoing Adipogenic Differentiation. *Tissue Engineering. Part C, Methods*, 25(6): 353-366.

Dheda, K., Huggett, J.F., Bustin, S.A., Johnson, M.A., Rook, G., Zumla, A. 2004: Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques*, 37(1): 112-4, 116, 118-9.

Eisenberg, E., Levanon, E.Y. 2013: Human housekeeping genes, revisited. *Trends in Genetics*, 29(10): 569-74.

Gabrielsson, B.G., Olofsson, L.E., Sjögren, A., Jernås, M., Elander, A., Lönn, M., Rudemo, M., Carlsson, L.M. 2005: Evaluation of reference genes for studies of gene expression in human adipose tissue. *Obesity research*, 13(4): 649-52.

Gorzelniak, K., Janke, J., Engeli, S., Sharma, A.M. 2001: Validation of endogenous controls for gene expression studies in human adipocytes and preadipocytes. *Hormone and Metabolic Research*, 33(10): 625-7.

Hurtado del Pozo, C., Calvo, R.M., Vesperinas-García, G., Gómez-Ambrosi, J., Frühbeck, G., Corripio-Sánchez, R., Rubio, M.A., Obregon, M.J. 2010: *IPO8* and *FBXL10*: new reference genes for gene expression studies in human adipose tissue. *Obesity*, 18(5): 897-903.

Mohammed, U., Samaila, M.O., Abubakar, M. 2014: Pattern of adipose tissue tumors in Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. *Annals of Nigerian Medicine*, 8(1): 8–10.

Omonte, S.V., de Andrade, B.A., Leal, R.M., Capistrano, H.M., Souza, P.E., Horta, M.C. 2016: Osteolipoma: A rare tumor in the oral cavity. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, 122(1): e8–e13.

Perez, L.J., Rios, L., Trivedi, P., D'Souza, K., Cowie, A., Nzirorera, C., Webster, D., Brunt, K., Legare, J.F., Hassan, A., Kienesberger, P.C., Pulinilkunnil, T. 2017: Validation of optimal reference genes for quantitative real time PCR in muscle and adipose tissue for obesity and diabetes research. *Scientific Reports*, 7(1): 3612.

Pérez, S., Royo, L.J., Astudillo, A., Escudero, D., Alvarez, F., Rodríguez, A., Gómez, E., Otero, J. 2007: Identifying the most suitable endogenous control for determining gene expression in hearts from organ donors. *BMC Molecular Biology*, 8: 114.

Radonić, A., Thulke, S., Mackay, I.M., Landt, O., Siegert, W., Nitsche, A. 2004: Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and Biophysical Research Communications*, 313(4): 856-62.

Schmittgen, T.D., Zakrajsek, B.A. 2000: Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *Journal of Biochemical and Biophysical Methods*, 46: 69-81.

Stojanović, S., Najman, S. 2019: The Effect of Conditioned Media of Stem Cells Derived from Lipoma and Adipose Tissue on Macrophages' Response and Wound Healing in Indirect Co-culture System *In Vitro*. *International Journal of Molecular Sciences*, 20(7): 1671.

Stojanović, S., Najman, S., Korać, A. 2018: Stem Cells Derived from Lipoma and Adipose Tissue—Similar Mesenchymal Phenotype but Different Differentiation Capacity Governed by Distinct Molecular Signature. *Cells*, 7(12): 260.

Suzuki, T., Higgins, P.J., Crawford, D.R. 2000: Control selection for RNA quantitation. *Biotechniques*, 29: 332-7.

Taube, M., Andersson-Assarsson, J.C., Lindberg, K., Pereira, M.J., Gäbel, M., Svensson, M.K., Eriksson, J.W., Svensson, P.A. 2015: Evaluation of reference genes for gene expression studies in human brown adipose tissue. *Adipocyte*, 4(4): 280-5.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A., Heinen, E. 1999: Housekeeping genes as internal standards: use and limits. *Journal of Biotechnology*, 75(2-3): 291-5.

Tricarico, C., Pinzani, P., Bianchi, S., Paglierani, M., Distante, V., Pazzagli, M., Bustin, S.A., Orlando, C. 2002: Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies.

Analytical Biochemistry, 309(2): 293-300.

Warrington, J.A., Nair, A., Mahadevappa, M., Tsyganskaya, M. 2000: Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. *Physiological Genomics*, 2: 143-7.

Zhang, W.X., Fan, J., Ma, J., Rao, Y.S., Zhang, L., Yan, Y.E. 2016: Selection of Suitable Reference Genes for Quantitative Real-Time PCR Normalization in Three Types of Rat Adipose Tissue. *International Journal of Molecular Sciences*, 17(6): E968.