Expression stability of ACTB, 18S, and GAPDH in human placental tissues from subjects with PCOS and controls: GAPDH expression is increased in PCOS

Authors

Evangelia Panagodimou[1], Vasiliki Koika[2], Fotios Markatos[1], Apostolos Kaponis[1], George Adonakis[1], Neoklis A Georgopoulos[2], Georgios K Markantes[2]

[1]Department of Obstetrics and Gynecology, University of Patras School of Health Sciences, Patras, Greece

[2]Division of Endocrinology - Department of Internal Medicine, University of Patras School of Health Sciences, Patras, Greece

Corresponding author: Georgios K. Markantes, Division of Endocrinology - Department of Internal Medicine, University of Patras School of Health Sciences, Patras, Greece, Tel.: 00302610999835, Email: gmarkantes@hotmail.com, ORCID: 0000-0003-1519-3776.

Abstract

Purpose: The aim was to assess the expression stability of three commonly used reference genes, namely, β-actin (*ACTB*), 18S ribosomal RNA (*18S*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in placental tissue obtained from pregnant women with polycystic ovary syndrome (PCOS) and healthy controls.

Methods: mRNA was isolated after delivery from the placentae of 10 PCOS and 10 control women with term, uncomplicated, singleton pregnancies. The expression of *ACTB*, *18S,* and *GAPDH* was analyzed using real-time polymerase chain reaction (RT-PCR). Gene expression stability was evaluated with the RefFinder, GeNorm, Normfinder, BestKeeper, and Delta-Ct tools.

Results: *ACTB* was ranked as the most stably expressed gene, followed by *18S*. The expression of *GAPDH* varied considerably in both studied groups, while it was increased in PCOS versus controls (5.3-fold, $p<0.05$).

Conclusions: *ACTB* is an appropriate reference gene for placental gene expression studies in women with PCOS, whereas *GAPDH* is unfit for such a role, as its placental expression is increased in PCOS.

Keywords: Polycystic ovary syndrome (PCOS), Placenta, Reference gene, Gene expression, RTPCR

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine abnormality in women of reproductive age, affecting up to 18% of these women depending on the criteria used for diagnosis. PCOS is the leading cause of anovulatory infertility and is also associated with increased risk of several adverse pregnancy outcomes, such as gestational diabetes, hypertension/preeclampsia, miscarriage, and preterm delivery [1]. Although the etiology of PCOS remains unclear, data from human and animal studies suggest that PCOS might begin in utero as a result of an adverse hyperandrogenic intrauterine environment [2]. The placenta, being the source of nutrients to the fetus as well as the main steroidogenic organ in pregnancy, has been implicated in the abovementioned theory concerning the pathogenesis of PCOS. Specifically, altered placental function has been shown in PCOS [3], while investigation of the molecular mechanisms involved is a topic of extensive ongoing research.

Quantitative real-time polymerase chain reaction (qRT-PCR) is one of the most common tools used to study the expression of genes in various tissues. This method provides a relative quantification of the target gene expression by comparing it to the expression of a “reference” or “housekeeping” gene (i.e., a gene that is considered to be constitutively expressed in the studied tissue). The ideal reference gene should be adequately expressed in the studied tissue and exhibit stable expression in all experimental conditions (i.e., in patients and controls), otherwise results might be spurious. Several studies have evaluated the suitability of traditional housekeeping genes to be used as reference for placental mRNA expression studies

[4,5]; results vary depending on the participants' characteristics (healthy placenta, preeclampsia, gestational diabetes, etc.). Particularly with respect to PCOS, data regarding appropriate reference genes are very limited [3,6].

In this study, we explored the placental expression stability of three commonly used reference genes, namely, β-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and 18S ribosomal RNA (*18S*) in pregnant women with and without PCOS.

Methods

Subjects and placental tissue collection

We used placental samples from 20 women participating in an ongoing prospective study comparing the placental expression of several genes in pregnant women with PCOS versus controls. The study was approved by the University Hospital of Patras Ethics Committee, Patras, Greece, and all participants provided written informed consent. Ten of the participants had PCOS (diagnosed before pregnancy according to the Rotterdam criteria) and 10 were healthy controls. All subjects had term, uncomplicated, singleton pregnancies and gave birth to healthy babies. Placental tissue was collected within 15min of placental delivery. Full-depth samples 1x1cm were excised from three areas at the middle point of the placental radius, away from obvious infarcts or damage. The maternal decidua and chorionic plate tissues were removed and each sample was further divided into 2-3 pieces of 0.5-1cm^3. Sampled tissues were thoroughly rinsed with saline to remove blood and were then

submerged in RNAlater solution and stored at 4^{o}C for 24 hours and then at -20^{o}C until analysis.

RNA extraction, reverse transcription, and real-time PCR

Isolation of total RNA from placental specimens was carried out, including a 15-minute DNAse I treatment, using the commercially available RNeasy Lipid Tissue Mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. RNA concentration and purity were estimated by measuring optical absorption at 260nm and calculating the ratio 260/280nm, respectively.

Complementary DNA (cDNA) synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (04379012001; Roche, Basel, Switzerland) with a mixture of anchored-oligo(dT)18 primer and 1μg of total RNA, according to the manufacturer's instructions.

qRT-PCR was performed in the LightCycler 2.0 Instrument (Roche, Basel, Switzerland), using 50ng of template cDNA and FastStart Universal SYBR Green Master (Roche Hellas). PCR primers can be provided upon request for the genes *ACTB* (NM_001101), *18S* (NR_003286), and *GAPDH* (NM_002046). Reactions were run in triplicates. The quality of the PCR reactions and specificity of the primers were confirmed by melting curve analysis. Relative gene expression was assessed using the ΔΔCt method.

Gene expression stability analysis

The RefFinder tool was used for the estimation of gene expression stability. RefFinder is a free web-based platform (https://www.heartcure.com.au/reffinder/)

which integrates the major currently available software packages (GeNorm, NormFinder, BestKeeper, and Delta-Ct method) to compare candidate reference genes in a given experiment. The RefFinder software generates a geometric mean (GM) value for each gene and ranks them in descending order from the most to the least stable. GeNorm and NormFinder provide a stability value (M) for each gene, the lowest value identifying the most stable gene [7, 8]. BestKeeper uses the standard deviation (SD) and coefficient of variation (CV) of the samples' Ct values, genes with low parameters being considered to have high stability. This software also provides a correlation coefficient (r) for each gene, with values close to 1 indicating high stability [9]. Finally, in the method proposed by Silver et al. [10], the mean delta-Ct and its standard deviation is calculated for each pair of candidate reference genes; the average standard deviation for each individual gene is used as a stability measure, with the lowest values corresponding to the most stable genes.

Statistical analysis

Parameters were tested for normality with the Kolmogorov-Smirnov test. Comparisons of gene expression between the groups were performed with the Mann-Whitney U Test. All procedures were performed using SPSS 25.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Demographics

Our study included 20 Caucasian pregnant women (10 with PCOS and 10 healthy controls). The mean ages of the two groups did not differ significantly (PCOS: 33.0±4.97 years, controls: 30.73±7.19 years, p=0.414).

Expression of reference genes

Using the RT-PCR cycle threshold value (Ct) to determine the expression level of the studied genes, we found that *ACTB* had the highest expression in both groups (Ct range: 16.41-22.98 in PCOS and 16.29-21.24 in controls), followed by *18S* (Ct range: 20.89-23.64 in PCOS and 19.65-23.83 in controls); *GAPDH* exhibited the lowest expression in PCOS (Ct range: 21.73-32.39) and in controls (Ct range: 24.24-33.98). Regarding SD of Ct values, *18S* had the lowest SD (1.16) in PCOS women, while *ACTB* and *18S* had similar SD (1.55 and 1.49, respectively) in controls. In both groups, *GAPDH* showed the greater dispersion of values (SD: 3.07 in PCOS and 3.06 in controls). Ct values were available for *ACTB* and *GAPDH* in all participants, and for *18S* in 10 subjects (four PCOS and six controls).

Gene expression stability

According to GeNorm, *ACTB* and *18S* had the best stability value (M=1.160), while *GAPDH* was the least stable gene (M=2.025). In the NormFinder analysis *ACTB* was ranked as the most stably expressed gene (M=0.580), followed by *18S* (M=1.592); *GAPDH* exhibited the highest variation (M=2.348) (Fig. 1A-1B).

Based on SD values calculated by BestKeeper, *18S* was the gene with the lowest variation (SD±CP=1.28, SD±x-fold=2.43) and, hence, the most stable; *ACTB* was the next most stable gene (SD±CP=1.44, SD±x-fold=2.71) and *GAPDH* was the least stable

(SD±CP=1.76, SD±x-fold=3.39) (Fig. 1C). However, based on the correlation coefficient, *ACTB* was the best reference gene (r=0.978, p=0.001). *18S* and *GAPDH* had lower r values (0.708, p=0.022 and 0.700, p=0.024, respectively).

According to the Delta-Ct method, *ACTB* presented the lowest variation in its expression (average SD=1.619). *18S* ranked second (average SD=1.998) and *GAPDH* was again the least stable gene (average SD=2.458) (Fig. 1D).

In the comprehensive gene stability analysis provided by RefFinder, *ACTB* was the best reference gene (GM=1.189), followed by *18S* (GM=1.414), while *GAPDH* exhibited the least stability among them (GM=3.000). The rankings performed by the aforementioned software tools are depicted in Table 1.

Relative expression of GAPDH in PCOS versus controls

Using *ACTB* as reference (as suggested by most gene stability software tools used), we assessed the relative expression of *GAPDH* in PCOS and healthy participants. We found that the placental expression of *GAPDH* was increased in PCOS (5.3-fold, $p<0.05$).

Discussion

In the present study, we investigated the placental expression of *ACTB*, *18S,* and *GAPDH* in women with and without PCOS. We showed that *ACTB* had the most stable expression and is a suitable reference gene for this setting. In contrast, *GAPDH*

is over-expressed in PCOS placentae and should not be used as reference in studies of placental gene expression including PCOS subjects.

In order to obtain reliable results in placental gene expression studies, it is necessary to use the appropriate reference genes. Although certain genes are traditionally used for normalization in RT-PCR experiments, it is evident that no single gene is constitutively expressed in all tissues and under all experimental conditions. Hence, assessment of the expression stability of potential reference genes is a prerequisite in such experiments.

A few studies have sought to identify appropriate reference genes for comparative expression studies in placental tissue. Results indicate that the expression stability of the tested genes may vary widely depending on the characteristics of the subjects included in each analysis. For example, *18S* and *GAPDH* were the most suitable reference genes in a study comparing placental gene expression in pregnancies with idiopathic fetal growth restriction and controls [5], but they were the least stable in other studies including pregnant women with diabetes and preeclampsia [4,11]. Similarly, *ACTB* performed well as a reference gene in certain studies [12], but not in others [5,11]. Even in studies including participants with similar characteristics results are not always consistent [4,11]. Consequently, reference gene stability should be analyzed for each tested experimental condition in any given experiment.

To our knowledge, there are no studies to date specifically designed to determine good reference genes for placental gene expression experiments in women with PCOS. In a study assessing placental STAT3 signaling in PCOS, Maliqueo et al. reported that the expression of *18S* and *ACTB* varied considerably between PCOS

and controls [3] but did not provide more information. In addition, *GAPDH* was used as internal control in a study profiling placental circular RNA expression in PCOS [6]. In our study, *ACTB* was ranked as the most stably expressed gene by almost all expression stability tools used. *18S* was identified as the most stable gene by BestKeeper, based on the SD values. However, it has been suggested that the correlation coefficient is a better parameter to assess the most stable genes when using this software [13]; considering the latter parameter, *ACTB* was again the most appropriate reference gene. Finally, *GAPDH* demonstrated considerable expression variability in both studied groups, while it was over-expressed 5.3-fold in PCOS relative to control placentae. *GADPH* is an enzyme participating in glycolysis, but also has non-metabolic functions (e.g., transcription regulation and intracellular protein transport) [4]. Studies in non-placental tissues have shown that *GAPDH* expression is induced by hypoxia, growth factors, cytokines, and also by insulin and glucose [14]. It is known that insulin resistance and hyperinsulinemia are very common in PCOS and could be exacerbated in pregnancy [1]. Furthermore, placental glucose metabolism and nutrient transport have been found to be altered in PCOS [3,15]. None of our PCOS subjects had frank diabetes or received insulin treatment. One woman with PCOS presented mild hyperglycemia in the second half of her pregnancy, but excellent glycemic control was achieved with diet only; notably, *GAPDH* relative expression in this subject was below the average of the whole PCOS group. Consequently, although aberrant glucose homeostasis might be an explanation for the increased placental expression of *GAPDH* in our PCOS subjects, other factors could also be involved. For example, it has been shown that the placenta in women with PCOS is often characterized by structural and functional abnormalities (vascular

lesions, reduced weight / thickness / volume, and increased androgen production) [16]; these alterations might be associated with chronic low-grade inflammation status (another feature of PCOS), which in turn could contribute to increased *GAPDH* expression via upregulation of proinflammatory cytokines [14,15]. Furthermore, the structural placental abnormalities observed in PCOS have been associated with impaired blood supply from the uterine artery [16]; this might lead to reduced oxygen supply and hypoxia, which is another potent *GAPDH* inducer [14].

The main limitation of our study is the small number of participants in the PCOS and control groups. More extensive studies are needed in order to corroborate our findings.

In conclusion, our study identified *ACTB* as an appropriate reference gene for relative gene quantification in placental tissue from PCOS women. *GAPDH* is unfit for such a role, as its placental expression is increased in PCOS.

Declarations - Compliance with ethical standards

Author contributions

Evangelia Panagodimou: Samples collection, critical revision, and editing of the manuscript.

Vasiliki Koika: Samples handling, gene expression analysis, and writing of the manuscript.

Fotios Markatos: Samples collection, critical revision, and editing of the manuscript.

Apostolos Kaponis: Samples collection, critical revision, and editing of the manuscript.

George Adonakis: Samples collection, critical revision, and editing of the manuscript.

Neoklis A Georgopoulos: Study design and writing of the manuscript.

Georgios K. Markantes: Study design, statistical analysis, and writing of the manuscript.

All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work.

Conflicts of interests

The authors have no relevant financial or non-financial interests to disclose.

Funding

This research is co-financed by Greece and the European Union (European Social Fund - ESF) through the Operational Programme "Human Resources Development, Education and Lifelong Learning 2014-2020" in the context of the project "Exploring the developmental theory for polycystic ovary syndrome: the role of alterations in placental gene expression and in fetal DNA methylation (MIS: 5047128)."

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the University Hospital of Patras Ethics Committee, Patras, Greece.

Informed consent - Consent to participate

All participants included in the study provided written informed consent.

Data availability

Available on request

References

[1] Yu HF, Chen HS, Rao DP, Gong J (2016) Association between polycystic ovary syndrome and the risk of pregnancy complications: A PRISMA-compliant systematic review and meta-analysis. Medicine (Baltimore) 95:e4863.

[2] Filippou P, Homburg R (2017) Is Foetal Hyperexposure to Androgens a Cause of PCOS? Hum Reprod Update 23:421–432.

[3] Maliqueo M, Sundström Poromaa I, Vanky E, Fornes R, Benrick A, Åkerud H, Stridsklev S, Labrie F, Jansson T, Stener-Victorin E (2015) Placental STAT3 signaling is activated in women with polycystic ovary syndrome. Hum Reprod 30:692-700.

[4] Meller M, Vadachkoria S, Luthy DA, Williams MA (2005) Evaluation of housekeeping genes in placental comparative expression studies. Placenta 26:601-607.

[5] Murthi P, Fitzpatrick E, Borg AJ, Donath S, Brennecke SP, Kalionis B (2008) GAPDH, 18S rRNA and YWHAZ are suitable endogenous reference genes for relative gene expression studies in placental tissues from human idiopathic fetal growth restriction. Placenta 29:798-801.

[6] Zhao C, Zhou Y, Shen X, Gong M, Lu Y, Fang C, Chen J, Ju R (2020) Circular RNA expression profiling in the fetal side of placenta from maternal polycystic ovary syndrome and circ_0023942 inhibits the proliferation of human ovarian granulosa cell. Arch Gynecol Obstet 301:963-971.

[7] Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.

[8] Andersen CL, Jensen JL, Orntoft TF (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 Res 64:5245-5250.

[9] Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechol Lett 26:509-515.

[10] Silver N, Best S, Jiang J, Thein SL (2006) Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol Biol 7:33.

[11] Lanoix D, Lacasse AA, St-Pierre J, Taylor SC, Ethier-Chiasson M, Lafond J, Vaillancourt C (2012) Quantitative PCR pitfalls: the case of the human placenta. Mol Biotechnol 52:234-243.

[12] Li Y, Lu H, Ji Y, Wu S, Yang Y (2016) Identification of genes for normalization of real-time RT-PCR data in placental tissues from intrahepatic cholestasis of pregnancy. Placenta 48:133-135.

[13] 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:e0122515.

[14] Suzuki T, Higgins PJ, Crawford DR (2000) Control selection for RNA quantitation. Biotechniques 29:332-337.

[15] Wang F, Xie N, Zhou J, Dai M, Zhang Q, Hardiman PJ, Qu F (2020) Molecular mechanisms underlying altered neurobehavioural development of female offspring of mothers with polycystic ovary syndrome: FOS-mediated regulation of neurotrophins in placenta. EBioMedicine 60:102993.

[16] Palomba S, Russo T, Falbo A, Di Cello A, Tolino A, Tucci L, La Sala GB, Zullo F (2013) Macroscopic and microscopic findings of the placenta in women with polycystic ovary syndrome. Hum Reprod 28:2838-2847.

Fig. 1 Gene expression stability analysis according to (A) GeNorm, (B) NormFinder, (C) BestKeeper, and (D) Delta-Ct methods. Low M, SD±CP, or Average SD values indicate high stability

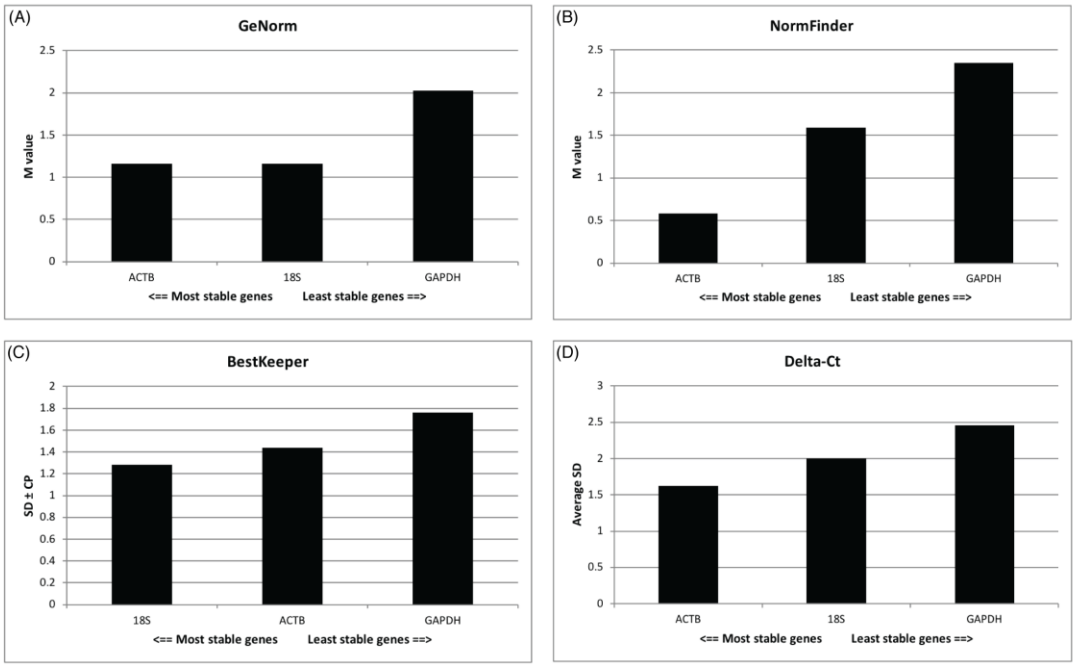

Table 1 Ranking order of the candidate reference genes by each of the software tools used

Rank	GeNorm	NormFinder	BestKeeper	Delta-Ct	RefFinder Comprehensive ranking
1	ACTB / 18S	ACTB	18S	ACTB	ACTB
2		18S	ACTB	18S	18S
3	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH