



Short communication

Evaluation of four commonly used normalizer genes for the study of decidual gene expression



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ABSTRACT

Reverse transcription quantitative PCR (RT-qPCR) gene expression results must be normalized using stably expressed genes to correct for technical variation. We evaluated the expression of four widely used normalizers (*RNA18S*, *GAPDH*, *TBP*, and *YWHAZ*) across 59 decidual tissue samples collected by vacuum suction from preeclamptic and normotensive pregnancies. *RNA18S* and *GAPDH* were not suitable as normalizers, while *YWHAZ* and *TBP* were stably expressed across the study groups.

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1. Introduction

Reverse transcription quantitative PCR (RT-qPCR) is commonly used to assay gene expression [1]. To correct for technical variation, RT-qPCR data must be normalized against genes (normalizers) which are stably expressed across study groups [2]. In the uteroplacental unit, RNA levels are dynamic, varying according to location [3], gestational age [4], and disease [5]. Therefore, for RT-qPCR studies of uteroplacental tissue, stable expression of normalizer genes across the relevant conditions must be tested.

Several studies have investigated normalizer genes in placental tissue from pregnancy complications and diabetes [6–8]. For decidual tissue from chorionic plate biopsies, Meller et al. evaluated potential normalizer genes in normotensive, hypertensive, and diabetic pregnancies [7]. We have previously established a vacuum suction method to collect decidual tissue during cesarean section

[9], but no studies have evaluated normalizers for this tissue, comparing preeclampsia and normotensive pregnancies.

In this study, we used existing gene expression data from third trimester decidual vacuum suction tissue to evaluate four commonly used normalizers for future preeclampsia studies.

2. Methods

Decidual samples were collected by vacuum suction as previously described [9,10]. Patient inclusion, and definition of preeclampsia is described in [10]. RNA isolation, cDNA synthesis, and qPCR (48-gene TLDA cards) were performed as described in [10]. All normotensive controls (n = 30) delivered after week 37, none demonstrated intrauterine growth restriction (IUGR). For the preeclampsia group (n = 29), nineteen were early-onset (delivery <week 34), 16 with IUGR, and ten late-onset (delivery ≥week 34), 4 with IUGR. IUGR was defined as <the 3rd gender-specific birth weight percentile and/or pathological umbilical artery Doppler measurement.

Expression results from the following assays are presented in this study; 18S ribosomal RNA (*RNA18S*) (Hs99999901-s1), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)

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(Hs99999905_m1), TATA-binding protein (*TBP*) (Hs99999910_m1), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*YWHAZ*) (Hs00237047_m1), and perilipin 3 (*PLIN3*) (Hs00998421_m1). The resulting cycle threshold (C_t) values from the RT-qPCR were transformed into relative quantities (RQs) and normalized RQs (NRQs) as described [11], assuming 100% amplification efficiency. Statistical significance was assessed using SPSS Version 22 (Mann-Whitney-Wilcoxon test).

3. Results

Among the four normalizers tested, *RNA18S* was the most highly expressed (lowest C_t values), followed by *GAPDH*, *YWHAZ*, and *TBP* (Table 1). *RNA18S* displayed the lowest variation (%CV) across all samples (Table 1), but with extreme values in both the control and preeclamptic groups (Fig. 1a) and non-significant increased expression in the preeclamptic versus the control group (Table 1 and Fig. 1a). *GAPDH* also displayed low variation across samples (Table 1), and significantly decreased expression in the preeclamptic versus control group (Table 1 and Fig. 1b). *TBP* and *YWHAZ* had similar expression levels between study groups (Fig. 1c and d), and higher variability than *RNA18S* and *GAPDH* (Table 1). Similar results were also found when analyzing the early and late-onset preeclampsia groups separately; however with a non-significant trend for *GAPDH* for late-onset preeclampsia relative to controls. The effect of each normalizer was examined using *PLIN3* as an example. Among the 44 genes on the TLDA card, *PLIN3* was selected for its moderate expression, low variability across samples, and no expression difference between controls and preeclampsia (Table 1 and Fig. 1e). With *RNA18S* as normalizer, two extreme data points were introduced (Fig. 1f), overall variability was increased (Table 1), and a significantly decreased fold-change in the preeclamptic versus control group was induced (Table 1). Normalization against *GAPDH* did not alter the data variability, but induced a significant fold-change increase in the preeclamptic versus control group (Table 1 and Fig. 1g). Normalization against *TBP*, *YWHAZ*, and their geometric mean did not introduce extreme data points nor induce significant fold-changes between the study groups (Table 1 and Fig. 1h–j).

4. Discussion

We have evaluated four commonly used RT-qPCR normalizer genes for the comparison of gene expression in vacuum suction decidual tissue from normal and preeclamptic pregnancies.

As previously reported, *RNA18S* was highly expressed in decidual tissue, and displayed a low variability across samples [4,12]. However, two samples had much lower expression levels than the rest, a trend also observed for the other tested genes (Fig. 1,

open data points) possibly reflecting technical issues affecting the qPCR reaction [2]. The enhanced effect on *RNA18S* expression could potentially reflect factors affecting mainly ribosomal/total RNA levels, with less effect on mRNA levels [13]. Off-target effects were excluded as a reported off-target transcript of the *RNA18S* assay; AK300665.1 (TNNC2, troponin C2) is not expressed in decidual tissue (microarray analysis, our unpublished results). Consequently, these extreme data points introduced extreme values in the *PLIN3* data normalized to *RNA18S*, which further induced a significantly decreased fold-change of *PLIN3* in the preeclamptic group relative to controls not observed for the other normalizers. Because of the high expression, which may affect expression level calculations [14], and presence of *RNA18S*-specific extreme data points, *RNA18S* was considered an unsuitable normalizer, in agreement with previous reports [13].

GAPDH, *TBP*, and *YWHAZ* exhibited more moderate expression levels than *RNA18S*. However, un-normalized *GAPDH* and *PLIN3* normalized against *GAPDH* displayed statistically significant fold-changes in preeclampsia relative to controls, a trend not observed for *TBP* and *YWHAZ*. Based on these results, *GAPDH* was considered an unsuitable normalizer for our study. Meller et al. [7] evaluated *GAPDH* as a normalizer in decidual tissue from normotensive and hypertensive pregnancies. High variability after normalization with *GAPDH* was observed, but evaluation of expression levels between study groups was not reported [7]. *GAPDH* expression can be affected by hypoxia, insulin and growth factors [15], factors relevant for the preeclamptic decidua, possibly explaining our observations. *TBP* and *YWHAZ* displayed stable expression across study groups and reduced the variability of *PLIN3* expression after normalization, with lowest variability achieved using their geometric mean, indicating their suitability as normalizers for decidual tissue, in agreement with [7].

Conflict of interest statement

The authors declare that they do not have any conflict of interest.

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Table 1
Data for evaluation of the possible normalizers. Median, minimum and maximum C_t , fold-change, p-value, and coefficient of variation are presented for each tested normalizer and *PLIN3*. Fold change, p-value and coefficient of variation are shown for each normalization of *PLIN3*. Fold-changes were calculated relative to controls; p-values were acquired using the Mann-Whitney-Wilcoxon test; coefficients of variation were calculated using all samples from all study groups.

	Median C_t (min-max)	Fold-change (FC)	p-value	Coefficient of variation (%CV)
RQ <i>RNA18S</i>	10.20 (9.64–15.77)	1.16	0.14	35.77
RQ <i>GAPDH</i>	22.58 (21.75–25.17)	0.82	0.01	36.04
RQ <i>TBP</i>	29.12 (28.15–31.38)	0.96	0.55	40.14
RQ <i>YWHAZ</i>	28.71 (27.29–31.85)	1.08	0.75	52.37
RQ <i>PLIN3</i>	26.05 (25.14–28.34)	0.99	0.57	33.32
NRQ <i>PLIN3</i> against <i>RNA18S</i>		0.91	0.02	181.43
NRQ <i>PLIN3</i> against <i>GAPDH</i>		1.22	0.00	34.03
NRQ <i>PLIN3</i> against <i>TBP</i>		1.03	0.58	26.50
NRQ <i>PLIN3</i> against <i>YWHAZ</i>		0.88	0.18	32.17
NRQ <i>PLIN3</i> against geometric mean of <i>TBP</i> and <i>YWHAZ</i>		0.96	0.38	21.96

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