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Research paper

Validation of RT-qPCR reference genes and determination of Robo4 expression levels in human retinal endothelial cells under hypoxia and/or hyperglycemia

Jia'nan Xie, Xin Liu, Ying Li, Yang Liu, Guanfang Su *

Department of Ophthalmology, Second Hospital of Jilin University, 218 Ziqiang Street, Changchun, Jilin, 130021, China

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ABSTRACT

Real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) has become the most common technique to investigate mRNA expression levels of target genes. In order to obtain accurate results, stable reference genes need to be selected for normalization in an experimental study. Human retinal endothelial cells (HREC) cultured in a hypoxic and hyperglycemic environment is a potential cell model to study diabetic retinopathy (DR), but the proper reference genes for RNA analysis have not yet been determined. In the present study, we evaluated the expression levels of 14 candidate housekeeping genes and selected the most suitable reference genes for RT-qPCR for HREC under hypoxic and/or hyperglycemic conditions. The results of the analyses using GeNorm, NormFinder, and BestKeeper software showed that a combination of *TBP*, *PUM1*, and *ALAS1* was most suitable for this research. Based on these results, mRNA expression levels of Roundabout4 (*Robo4*) in HREC were determined. The RT-qPCR analysis showed that there was a significant increase in *Robo4* expression under hyperglycemic conditions, while there was a decrease in expression under hypoxic and combined hypoxic and hyperglycemic conditions, suggesting that Robo4 might play different roles in various stages of DR.

1. Introduction

With the advantages of high sensitivity and potential for absolute quantification of transcripts, real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) has become the most common technique for gene expression analysis (Bustin et al., 2005). RT-qPCR needs to be normalized by reference genes, and the selection of appropriate endogenous controls is essential to obtaining accurate and reliable results. Housekeeping genes, which are indispensably expressed in all types of cells to maintain essential cell functions, are widely used as reference genes in RT-qPCR (Kozera and Rapacz, 2013). However, increasing evidence indicate that the expression of housekeeping genes can be variable, particularly in different tissues and cells or under various treatment conditions (Schmittgen and Zakrajsek, 2000; Stern-Straeter et al., 2009; Greer et al., 2010). This suggests that no housekeeping gene is suitable for all experimental conditions. Therefore, in different investigations of gene expression, it is important to evaluate the stability of housekeeping genes and choose the ideal reference gene in order to ensure that RT-qPCR results are accurate (Dheda et al., 2004).

Human retinal endothelial cells (HREC) are major components of the inner blood-retinal barrier (Korte et al., 1984; Cunha-Vaz et al., 2011), playing essential roles in preventing the leakage of materials into retinal vasculature and maintaining retina function (Tong et al., 2013; Deissler et al., 2014). Breakdown of the blood-retinal barrier can lead to multiple retinal vascular diseases; diabetic retinopathy (DR) is the most common of these (Zhang et al., 2014). Several studies have demonstrated that the high glucose levels in early-stage DR are intimately involved in HREC dysfunction (Trudeau et al., 2010), which can cause an increase in retinal vessel permeability, leakage of harmful substances, and degeneration of capillaries, finally resulting in hypoxia in the retina (Arden and Sivaprasad, 2011; Chronopoulos et al., 2011). In the pathological process of DR, hyperglycemia and hypoxia may interact and may both contribute significantly to the progression of DR (Nyengaard et al., 2004). Many studies analyzing gene expression by RT-qPCR in HREC under hyperglycemic and hypoxic conditions has been reported in recent years (Rangasamy et al., 2011; Tian et al., 2015); however, the reference genes for human retinal endothelial cell assays have not always been properly validated.

Abbreviations: RT-qPCR, real-time reverse transcription quantitative polymerase chain reaction; HREC, human retinal endothelial cells; DR, diabetic retinopathy; Robo4, roundabout4; RPE, retinal pigment epithelium; PDR, proliferative diabetic retinopathy; 18S, 18S ribosomal RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; B2M, beta-2-microglobulin; ACTB, actin, beta; ALAS1, 5′-aminolevulinate synthase 1; GUSB, glucuronidase, beta; HPRT1, hypoxanthine phosphoribosyl transferase 1; HMBS, hydroxymethylbilane synthase; PPIA, peptidylprolyl isomerase A; PUM1, pumilio RNA binding family member 1; RPL29, ribosomal protein L29; TBP, TATA-box binding protein; RPLP0, ribosomal protein, large, P0; TFRC, transferrin receptor.

* Corresponding author.
E-mail address: sugf2012@163.com (G. Su).

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Here, we used RT-qPCR to evaluate the expression levels of 14 candidate housekeeping genes, including *18S*, *GAPDH*, *B2M*, *ACTB*, *ALAS1*, *GUSB*, *HPRT1*, *HMBS*, *PPIA*, *PUM1*, *RPL29*, *TBP*, *RPLP0*, and *TFRC* under hypoxic and/or hyperglycemic conditions in HREC. We also validated these potential reference genes using statistical programs, including GeNorm, NormFinder, and BestKeeper (Wang et al., 2012; Ali et al., 2015). In addition, based on the chosen reference genes, we investigated the expression of Roundabout4 (*Robo4*), a specific gene affecting the permeability of vascular endothelial cells (Jones et al., 2008), of HREC in a simulated DR environment to demonstrate the role of this gene in DR generation and development.

2. Materials and methods

2.1. HREC culture

HREC were kindly donated by Professor Liu of Tongji University and cultured in Endothelial Cell Medium (Invitrogen) with 5% fetal bovine serum (Invitrogen) and 1% Endothelial Cell Growth Supplement (Bio Co., China) at 37 °C in an incubator with 5% CO_2.

2.2. Hypoxia and/or hyperglycemia treatment

HREC were treated with four different conditions: cells cultured in normoxic (21% O_2) and normoglycemic (5.5 mM glucose) medium as the control; cells in normoglycemic medium were incubated for 1, 2, or 3 days in a sealed and anaerobic workstation (Ruskin Technologies, Pencoed, Wales, UK) with 1% O_2, 5% CO_2, and 94% N_2, and the same temperature (37 °C) and humidity (90%) as the hypoxic condition; in the hyperglycemic condition, high glucose (25 mM glucose) medium was used for 3, 5, or 7 days of culture in a normoxic atmosphere; in the combination of hypoxic and hyperglycemic conditions, HREC were cultured with high glucose and placed in the sealed and anaerobic workstation for up to 3 days. A summary of the eight different experimental groups is presented in Table 1.

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted from HREC using an RNA isolation kit (Dingguo Bio, Inc., China) according to the manufacturer's protocol. The concentration and purity of the RNA were confirmed by measuring the absorbance at A260/A280 using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A ratio of A260/A280 of approximately 2.0 was generally accepted as pure RNA. The integrity of RNA samples was assessed using 1% agarose gel electrophoresis. Then, 600 ng of RNA from each sample was reverse transcribed to synthesize cDNA using an RT Reagent kit (Takara Bio, Inc., China) according to the manufacturer's instructions. A 20 μL volume of cDNA was stored at −20 °C for RT-qPCR analysis.

2.4. Quantitative real-time PCR

Sequences of primers for the 14 candidate reference genes and *Robo4*, procured from Sangon Company (Shanghai, China), are listed in Table 2. The final reaction mixture was 20 μL, containing 1 μL cDNA, 8 μL tri-distilled water, 10 μL of FastStart Universal SYBR Green Master (Roche Diagnostics, Basel, Switzerland), and 10 μM each of the forward and reverse primers. Quantitative PCR was carried out in triplicate in 96-well optical reaction plates using a LightCycler 480 (Roche) under these amplification conditions: an initial step of 94 °C for 3 min to activate the polymerase, followed by 40 cycles of 94 °C for 20 s, 57 °C for 20 s, and 72 °C for 20 s for DNA denaturation, annealing, and elongation, respectively. The melting curve analysis was performed after each amplification by heating to 95 °C to assess the specificity of products. A total of 8 cDNA samples in different experimental conditions were amplified at the same time. The cycle threshold (Ct) values were calculated using LightCycler 480 software.

The gene expression levels of the 14 candidate reference genes were calculated and their gene expression stabilities were compared using the GeNorm, NormFinder and BestKeeper programs. Changes in mRNA expression levels of *Robo4* in HREC were analyzed under hypoxic and/or hyperglycemic conditions relative to those from cells in control conditions using the GeNorm and delta–delta Ct methods, normalized by the selected stable reference genes.

Table 1
HREC sample groups according to experimental conditions.

	G1	G2	G3	G4	G5	G6	G7	G8
Normoxia (21% O_2)	+				+	+	+	
Hypoxia (1% O_2)		1 d	2 d	3 d				3 d
Normoglycemia (5.5 mM)	+	+	+	+				
Hyperglycemia (25 mM)					3 d	5 d	7 d	3 d

HREC were divided into eight groups (G). G1 was the control group, G2 to G4 were the hypoxia groups, G5 to G7 were the hyperglycemia groups, and G8 was the combined hypoxia and hyperglycemia group.

2.5. Statistical analysis

All experiments were repeated three times. SPSS software was used to analyze the mRNA expression of the target gene in all experimental groups using Student's *t*-test, with the significance level (P) set at 0.05.

3. Results

3.1. Specificity and efficiency of the primers

The products of quantitative PCR were detected by electrophoretic analysis. Amplified fragments of all reference genes were consistent with the expected length, demonstrating that there was no nonspecific amplification, and the melting curves showed a single peak, confirming the specificity of the primers (data not shown). The primer efficiencies, determined by curves generated for serial dilutions of genes, ranged from 91% to 110%, and correlation coefficients were greater than 0.95.

3.2. Expression of reference genes

RT-qPCR was performed and the relative expression value for each reference gene in HREC cultured in different conditions was analyzed by the $2^{-\Delta CT}$ method, as presented in Fig. 1. Our results showed that expression levels of some reference genes varied greatly among the different conditions. The *TFRC* gene was found to be stably expressed with hypoxia for 1 day compared to the control, but downregulated with hypoxia for 2 days and significantly upregulated after 3 days. Under hyperglycemic conditions, the expression of *TFRC* was obviously downregulated again. The expression levels of Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*), which are the most commonly used genes for normalization, ranged widely under hypoxic and/or hyperglycemic conditions, suggesting that *GAPDH* and *ACTB* is variable and they were not suitable to serve as endogenous controls in the present experiment. In contrast, *PUM1* and *TBP* displayed stably lower expression under hypoxic and/or hyperglycemic conditions in all intervals compared to the control.

3.3. Variation in reference genes and optimal number for normalization

For the selection of housekeeping genes, GeNorm analysis software was used first. The core principle of the GeNorm program is that the expression ratio of two stable housekeeping genes should be consistent across all samples (Vandesompele et al., 2002), and that greater variability in the expression ratio signifies less stable reference genes. It provides a method to rank gene stability with a gene expression stability value (M), which is calculated as the average pair-wise