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Original article

Selection of reliable reference genes in eutopic and ectopic endometrium for quantitative expression studies

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ABSTRACT

Purpose: Physiological changes during menstrual cycle cause the endometrium and endometriosis to develop specific kind of tissues, especially in regard to the gene expression profiles, which may include also housekeeping genes, commonly used as reference genes (RGs) in quantitative studies. Reverse transcription, followed by quantitative polymerase chain reaction (RT-qPCR) is the most precise and commonly used method in gene expression studies. In order to reduce effects of technical approaches and biological variability of gene's expression level, the studies often employ RGs in experimental data normalization. However, the expression of RGs is not always stable and depends on several variables. Thus, the selection of appropriate RG is one of the most significant steps to obtain reliable results in RT-qPCR-based methods.
Material and methods: With the usage of RT-qPCR, we researched the expression of seven genes (*ACTB*, *B2M*, *G6PD*, *GAPD*, *GUSB*, *HPRT* and *PPIA*) as reliable reference genes in eutopic and ectopic endometrial tissue specimens obtained during standard surgery of women of reproductive age. Stability of expression level was analyzed by the most universal MS Excel plug-ins including: geNorm, NormFinder and BestKeeper. The descriptive statistics were evaluated using Statistica software.
Results: The distribution of threshold (C_t) values was not equal. We identified genes with higher expression level (referring to C_t values) such as *ACTB* and *B2M*, medium e.g., *GAPD* and low expression level, e.g., *G6PD* and *HPRT*. We demonstrated that the stability of the analyzed reference genes was not homogenous, and different algorithms pointed to *PPIA*, *GAPD* and *B2M* as the most stable ones in eutopic and ectopic endometrium. On the contrary to these, *GUSB* and *G6PD* were the most unstable ones.
Conclusions: In RT-qPCR-based analyses of gene expression level in eutopic and ectopic endometrium, we strongly recommend that a minimum of two reference genes are to be used and we determined that the most suitable seem to be *PPIA* and *GAPD*.

1. Introduction

Endometrium is particularly specific kind of tissue. Its periodic alterations require proper interaction between many factors. Expression of various genes depends on the menstrual cycle phase. Expression of genes relies not only on physiological changes which occur during menstrual cycle but also on several gynecological pathologies, such as endometrial cancer and endometriosis which affect the genes' expression, also potentially including the so called housekeeping genes (HKGs). Endometriosis (ectopic endometrium) is a chronic disease characterized by implantation and overgrowth of endometrial cells outside the uterine cavity. Eutopic and ectopic cells demonstrate functional similarity but additionally manifest significant structural and molecular differences. Endometriosis can lead to dysfunction of the female reproductive system and underlie fertility disorders. Pathogenesis of infertility in endometriosis is based on pathological changes of the ovaries and the fallopian tubes as well as on influence of the hormonal, biochemical and immunological alterations in the eutopic endometrium [1–4]. Multiple factors have so far been implicated and pathogenesis of endometriosis

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continues to be discussed. Although eutopic endometrium seems to have an important role in the physiopathology of the disease, the origin of this disease still remains unknown [5]. Thus, the last few years have seen the appearance of increasing number of papers on topic of the genetic and epigenetic determinants of endometriosis. Due to presence of a substantial number of factors, which modulate physiology of endometrium, quantitative analyses in gene expression studies encounter many problems, including the difficulty of selecting appropriate reference genes (RGs) [6,7].

Reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR) is the most scrupulous and commonly used method for a gene of interest (GOI) expression study. The main advantages of this technique include high sensitivity and reliability along with fast rate of receiving the results and wide range of applications. These features have resulted in RT-qPCR outclassing other, conventional methods of measuring gene expression [8]. Two different ways in which level of RNA expression can be measured exist. The first is the absolute quantification method which generates a standard curve. It is calculated by comparing GOI with the known number of copies to the sample where the level of transcript is unknown. The second one, which is the relative quantification allows analysis of gene expression by normalization of the fluorescence signal of investigated sample to internal control reference genes, the expression of which is considered to be constitutive under various experimental conditions [8,9]. As a matter of fact, the expression of RG is not always stable and depends on various factors given that small changes in technical factors may have extensive effects on experimental outcomes. Therefore it is crucial for qPCR data to be normalized in order to reduce this variability. However, normalization of relative quantification may provide obstacles, and choice of appropriate internal control gene needs to be determined empirically, especially if small changes in gene expression are expected. Hence, the selection of appropriate RG is one of the most important steps to obtain reliable results in RT-qPCR-based methods. Accurate quantitation of gene expression levels using RT-qPCR is highly dependent on normalization of the GOI against the most suitable reference gene and following the "Minimal Information for the Publication of real-time PCR guidelines" (MIQE), more than one RG is required for optimum normalization during RT-qPCR. Normalization against a single reference gene should not be acceptable unless a clear evidence is provided which confirms its stable expression level under changing physiological and experimental conditions [10].

The most commonly used reference genes in quantitative PCR-based methods belong to the housekeeping genes (HKGs) which are the large group of genes encoding proteins responsible for maintenance of basic cellular functions. In addition, the genes should manifest constant expression in all cells and tissues of the body, independently of its physiological or pathophysiological condition [11]. There are many HKGs/RGs which are used in gene expression studies. Genes such as those coding for beta-actin (*ACTB*), glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1 (*HPRT*), beta-2-microglobulin (*B2M*), glucose-6-phosphate dehydrogenase (*G6PD*), beta-D-glucuronidase (*GUSB*), peptidylprolyl isomerase A (*PPIA*) and other, by way of their crucial role in cell function and constitutive nature are the most frequently used reference internal controls [12].

However, several studies have proved that expression of the commonly used reference genes, including the above-mentioned ones, did not remain constant under many variables, such as distinct kind or condition of tissue and metabolic state [8,12–14]. Regarding the MIQE guidelines, the RGs used in experimental models should be met with some specific requirements [10]. Most importantly, their expression should not be affected by tentative conditions and remain constant, regardless of the type of examined tissue. Moreover, detection of fluorescence signal has to be specific only for RNA. Primers should be properly and carefully designed in order to avoid DNA and pseudogene amplification, which might interfere with the results [15,16].

The choice of proper internal control gene is crucial for normalizing RT-qPCR data obtained during normalization GOI against RGs and leading to reduction of possible gene quantification errors and sample to sample variability [17,18]. Expression of HKGs/RGs may be influenced by many factors, such as nutritional state, tissue pathogenesis, systemic or local disease and experiment validation [19–21].

In this study we investigated the expression of housekeeping genes as reliable reference genes in eutopic and ectopic endometrial tissue specimens obtained during standard surgical procedures of women of reproductive age.

2. Material and methods

Eutopic and ectopic endometrial tissue specimens were obtained from 188 females of Western European descent (51 non-pathological endometrial tissue samples vs. 137 pathological). Later it was limited to 23 cases because of strict selection criteria applied to the analysis (e.g., the rate of decay of mRNA for majority of genes is not known [22], and quality and quantity of RNA can vary among samples regarding to the time and storage conditions and the tissue itself [14]). Non-pathological endometrial tissue samples ($n = 13$) were collected from patients having a surgery for reasons other than endometriosis, during standard hysterectomy (diagnosed with cervical intra-epithelial neoplasia). The pathologically altered tissue fragments ($n = 10$) were acquired from patients during laparoscopy or laparotomy in the Department of Mother's and Child's Health, Poznan University of Medical Sciences. The age of patients ranged from 28 to 42 years. Endometriosis was confirmed by histopathological macroscopic and microscopic examinations. All patients had not been treated by hormonal therapy for at least 6 months before the surgery and showed regular menstrual cycles. Every specimen of tissue was obtained during the mid-secretory phase of menstrual cycle which was confirmed by the measurement of serum hormone levels. The material was used for epithelial cell isolation, as well as for histological and immunohistochemical analysis. Immediately after the surgery, the tissue specimens were placed in RNA protection buffer, RNA *Later* buffer (Sigma–Aldrich, St. Louis, MO, USA) at −80 °C until isolation of genetic material was conducted.

The study protocol was approved by the Institutional Review Board of the Poznan University of Medical Sciences (No 163/08). All patients provided written informed consent to participate in this study under a protocol approved by the Local Ethics Review Board of Poznan University of Medical Sciences.

2.1. RNA isolation and reverse transcription

Total cellular RNA was extracted using TriPure Isolation Reagent (Roche Diagnostic GmbH, Mannheim, Germany), according to manufacturer's protocol. The procedure was carried out twice. The concentration of total RNA was determined spectrophotometrically (NanoDrop ND-1000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA). Denaturing agarose gel electrophoresis was carried out to check integrity of the RNA. Only those samples were used, where the total RNA concentration ranged from 1 μg/μl to 2 μg/μl, and the OD(260/280) and OD(260/230) ranged from 1.8 to 2.0 (1.91 ± 0.039) and from 1.9 to 2.0 (1.94 ± 0.054), respectively. The integrity was confirmed electrophoretically in 1.2% agarose gel containing 1.5% formaldehyde (Sigma–Aldrich, USA) in FA buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 200 mM