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Research Article

Appropriate Reference Gene for the Gene Expression Analysis in U87 Glioblastoma Cell Line

Mina Lashkarboloki[1], Amin Jahanbakhshi[2], Seyed Javad Mowla[1], Bahram M. Soltani[1*]

[1] *Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran*
[2] *Stem Cell and Regenerative Medicine Research Centre, Iran University of Medical Sciences (IUMS), Tehran, Iran*

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Abstract

Cancer is one of the most challenging diseases in the world. It is widely accepted that knowing the molecular aspects of diseases, including cancers, helps to develop methods for their therapy and diagnosis. Long non-coding RNAs (lncRANs) are a novel category of regulatory genes known to be involved in cancer incidence. The expression of these genes is said to be suitable of using in prognosis, diagnosis, targeted therapy, etc. The RT-qPCR method that is widely used for analyzing the gene expression requires the application of appropriate reference genes as the internal control. The expression status of a proper housekeeping reference gene is not supposed to change under experimental circumstances. This study aimed to find a suitable reference gene in the U87 cells after overexpression of a gene of interest. To this aim, the expression status of four common reference genes (*ACTB*, *β2M*, *GAPDH*, and *HPRT1*) was examined in the transfected U87 cells. The U87 cells were transfected with a vector overexpressing YWHAE-lncRNA and an empty vector (mock). After total RNA extraction and cDNA synthesis, RT-qPCR was applied using the aforementioned internal control genes. Data were analyzed, and their graphs were plotted in GraphPad Prism 8.2 software. *B2M* showed the most change; accordingly, *GAPDH and HPRT1* expression levels were changed about 5 and 4 times, respectively. Of the candidate genes, only the *ACTB* gene had a consistent expression level in two different modes of transfection, and therefore, it is suggested as an appropriate reference gene for the study of gene expression in the transfected U87 cell line. It is remained to be tested if *β2M*, *GAPDH*, and *HPRT1* common internal controls are specifically affected by YWHAE-lncRNA overexpression or other lncRNAs may affect their expression as well.

***Keywords*:** Long non-coding RNA, Housekeeping genes, Real-Time PCR

Introduction

Glioblastoma multiform (GBM) is the most common type of malignant tumor in the central nervous system with low survival (Louis et al., 2007). Despite many scientific and medical advances in recent decades, there is still insufficient information on molecular pathogenesis and complex intracellular biological interactions that regulate the progression of the disease (Appin et al., 2014). Therefore, it is necessary to identify critical molecular pathways involved in the development and progression of glioma to provide new biomarkers for improvement or treatment. Genetic changes initiate various biological processes, leading to many disorders and tumor formation. Many types of research have focused on the molecular differences between tumors and normal tissues (Futreal et al., 2004; Hornberg et al., 2006). Analysis of gene expression using real-time quantitative PCR is a standard approach to identifying genes with different expression levels (Akiyama et al., 2014). RT-qPCR is a highly sensitive, specific, and reproducible method for obtaining validated results (Taylor et al., 2019). Different factors such as the amount of raw material, RNA quality and quantity, cDNA synthesis efficiency can affect gene expression results in the qPCR method (Nolan et al., 2006). Therefore, real-time data must be normalized with a suitable internal control gene (Wong and Medrano, 2005; Soltanian et al., 2021). Appropriate reference genes should

*Corresponding author's e-mail address: *soltanib@modares.ac.ir*

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have a consistent expression level in all samples, regardless of tissue type, growth stage, disease status, and pharmacological or biological treatments. For example, *GAPDH* is reported as a suitable reference gene in nano-curcumin-treated colorectal cancer-originated cells (Choori et al., 2018). It is widely accepted that the expression of no gene is stable in all the cellular conditions and therefore the proper candidate reference genes must be validated under each experimental condition (Dundas and Ling, 2012). Several studies introduced a set of reference genes for the study of glioma tissue, but their results were not consistent. Therefore, they suggested that in each study, a suitable reference gene should be approved (Kreth et al., 2010). In the present study, we investigated the suitability of four reference genes, *ACTB, β2M, GAPDH,* and *HPRT1*, for qPCR analysis in the human glioma U87 cell line.

Materials and Methods

Cell Culture and Transfection

The U87 cell line (ATCC: HTB-14) was provided from the the Pasteur Institute Cell Bank (Tehran, Iran) and cultured in HG-DMEM (Gibco) medium. The culture medium was supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/mL of penicillin, 100 µg/mL of streptomycin (Sigma-Aldrich). Incubation was performed at 37 °C with 5% CO_2, and the cells with 85 to 90% confluency were equally seeded in a 24-well plate. The cells were transfected with the plasmid containing YWHAE long non-coding RNA gene, after 24 hours. Transfection was performed using TurboFect reagent (Thermo Fisher Scientific, USA) according to manufacturer protocol. PCDNA3.1 plasmid without fragment was transfected as the mock control. By using a reverse fluorescent microscope (Nikon eclipse Te2000-s, Japan) GFP signal was observed 24 hrs after transfection to measure the rate.

RNA Extraction and cDNA Synthesis

Total RNA was extracted using RiboEx (Krishgen Biosystems, India), according to the manufacturer's protocol. Agarose gel electrophoresis and NanoDrop TM 1000 (Thermo Scientific, USA) were used to measure the quality and quantity of the extracted RNA, respectively. Depending on the quantity and quality of RNAs, different volumes but the same concentration of RNAs (1µg/µL) were used for cDNA synthesis. The cDNA synthesis was performed using ExcelRT™ Reverse Transcriptase (SMOBIO, Taiwan) and Oligo $(dT)_{18}$ and Random hexamers as primers.

RT-qPCR

In order to evaluate the quality of cDNA synthesis, PCR reactions were performed in 28 cycles using *β2M, GAPDH, HPRT1,* and *ACTB* gene primers for each group of synthesized cDNAs. Then, qPCR was carried out using 50 ng concentration of cDNA, SYBR Premix Kit (TaKaRa, Japan), and specific primers for considered reference genes in StepOne™ Real-Time PCR.

Statistical Analysis

The qPCR data were analyzed according to the $2^{-\Delta\Delta Ct}$ methods. Then, GraphPad Prism software (version 6) was used to perform the t-test and draw the charts. Results with a *p*-value less than 0.05 were considered statistically significant.

Results and Discussion

Transfection

The transfection rate of U87 cells was 50% which was examined and confirmed by pEGFP-C1 as a control vector (Figure 1A). Also, to ensure the transfection efficiency, the expression of the lnc-YWHAE gene was investigated by qPCR in overexpression construct and empty construct (mock). The results showed that lnc-YWHAE expression in overexpressed cells increased 43,000 times (CT 32 reduced to CT 16) compared to the control cells (Figure 1B).

RNA Extraction and cDNA Synthesis

Assessment of the quality of RNA by agarose gel electrophoresis and NanoDrop showed that RNA extraction from each sample has an acceptable quality, but these RNAs are not of the same quantity (Figure S1). However, RNA with a final concentration of 1 µg was used for cDNA synthesis. After cDNAs were synthesized, to confirm their quality, PCR reactions were performed in 28 cycles for the *β2M* gene. The agarose gel electrophoresis showed that the cDNAs had been synthesized with good quality, but their quantity is different from each other (Figure S2).

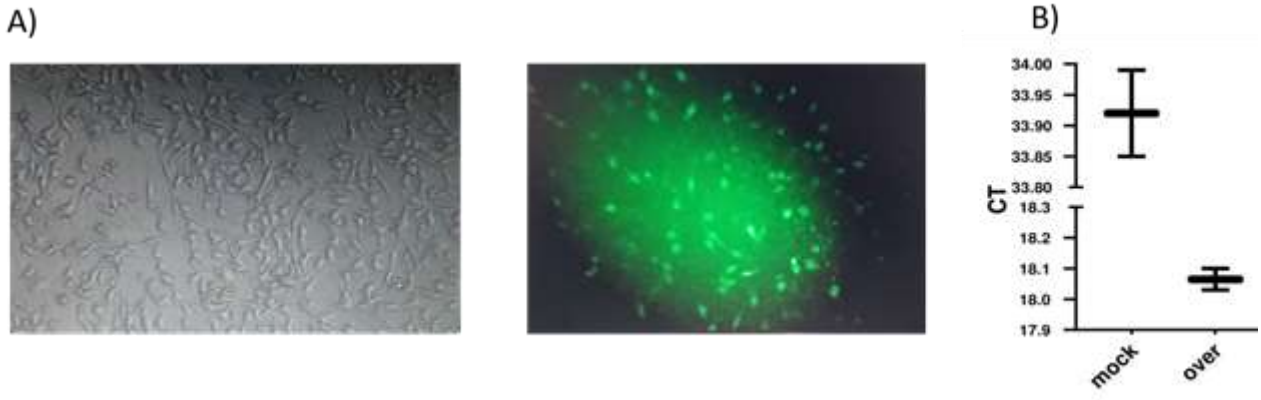

Figure 1. The transfection rate. A) Fluorescent microscope image shows that the structure transfers to the u87 cell line. B) To confirm transfection, lnc-YWHAE expression was measured in controlled and over-expressed cells. Lnc-YWHAE CT was reduced to 18 in overexpressed samples.

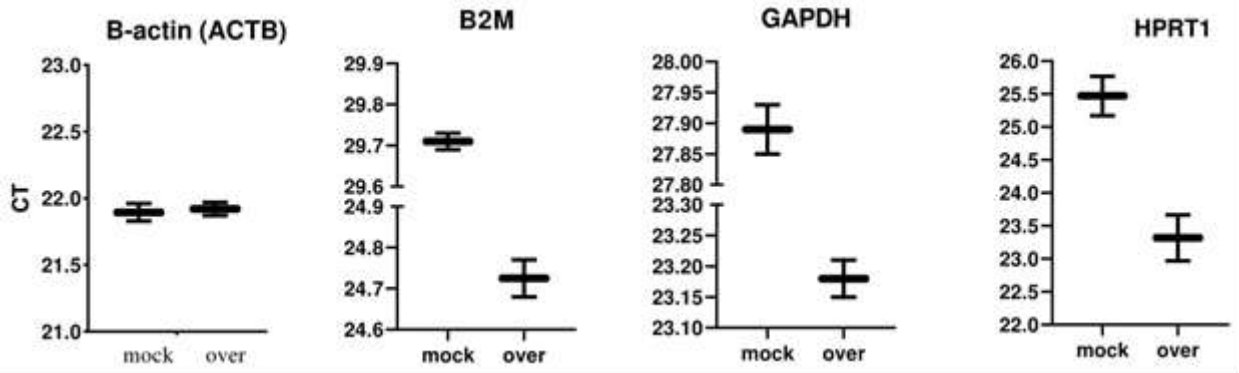

Figure 2. Ct of the reference gene. RT-qPCR results show that the expression of all housekeeping genes except *B-actin* (*ACTB*) changes after overexpression of the target gene.

RT-qPCR

One ng of cDNA synthesized was used in the qPCR reaction for four typical reference genes *ACTB*, *β2M*, *GAPDH*, and *HPRT1*. Specific amplification was confirmed by a single peak melting curve diagram (Figure S3). Results showed that Ct values of *β2M*, *GAPDH*, and *HPRT1* genes in sample 1 (U87 transfected with YWHAE long non-coding RNA) and sample 2 (U87 transfected with empty construct) alter, but for *ACTB*, the threshold cycles are very close and almost identical (Figure 2). Moreover, statistical analysis revealed that if the *ACTB* gene is used as a reference gene, *β2M*, *GAPDH*, and *HPRT1* gene expression will alter after the transfection.

Recent studies on glioma introduced various genes as internal controls. The *GAPDH*, *RPL13A*, *CYC1*, and *ACTB* were used as the housekeeping genes in real-time PCR data analysis on glioma tissue samples (Grube et al., 2015; Röhn et al., 2018). One of the effective factors in gene expression is the process of tumor progression. The *GAPDH*, *IPO8*, *RPL13A*, *SDHA*, and *TBP* genes were suitable internal controls that did not change in different grades (II-IV) of human astrocytoma, and *TBP* and *IPO8* were the most stable of them (Kreth et al., 2010; Gabriele et al., 2018). But in another study, the *RPL13A* and *TBP* were stable in all of the samples and ideal for analyzing gene expression in glioblastoma, whereas other genes, such as *GAPDH* and *β2M*, were not suitable due to their changes in RNA expression levels (Aithal and Rajeswari, 2015). Some studies reported that *HPART* is a suitable reference gene, while others do not (Kreth et al., 2010; Valente et al., 2014; Aithal and Rajeswari, 2015). However, the effects of *in vitro* manipulation of cells, such as drug treatment or gene transfection, on the conditions are very variable.

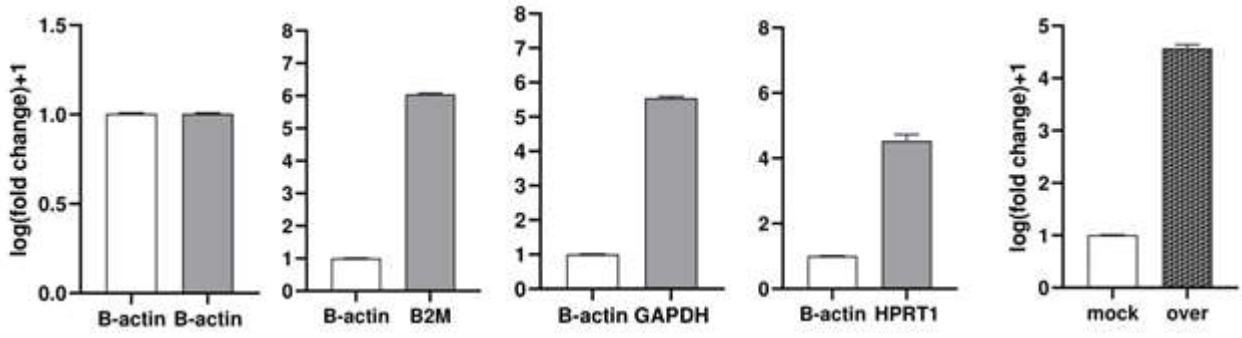

Figure 3. Lnc-YWHAE normalization after selection of the suitable internal control. A) Altered expression of all the internal control genes except β-actin after normalization with internal control of β-actin. B) Measurement of lnc-YWHAE expression level after normalization with β-actin shows the overexpression of this gene after transfection.

After transfection of long non-coding RNAs, such as *HOTAIRM1*, *SUMO1P3*, and *AC003092.1*, to the U87 cell line and their overexpression, *GAPDH* has been used for normalization (Xu et al., 2018; Lin et al., 2020; Lou et al., 2020). But in the other study, *ACTB* was selected as a reference in the U87 cell line (Li et al., 2020; Nie et al., 2015; Jung et al., 2013). Also, in other glioma cell lines, U251 and A172, *GAPDH* has been used (Liu et al., 2017; Wang et al., 2019). So, in different studies, the appropriate reference genes are different.

Our study showed that in order to select reliable reference genes, the specific conditions of each experiment must be considered. In order to achieve this possibility, first ,the quantity and quality of RNAs were determined, and then, cDNA synthesis was performed. After examining the quality of synthesized cDNAs by 28-cycle RT-PCR, the real-time-qPCR reaction was performed. Among the four genes, only *ACTB* expression did not change and was constant under different cell transfection conditions, while other genes, *β2M*, *GAPDH*, and *HPRT1*, were not stable (Figure 3A). Overexpression of YWHAE long non-coding RNA affects most internal control gene expression. Many studies have used a typical housekeeping gene as a reference gene, but our results show that in addition to using experiences, specific conditions of the test, type of samples, cell lines, and gene structure transfected into cells must be considered. For this purpose, before starting the analysis of RT-qPCR data, a suitable reference gene should be selected.

In this study, we first showed that YWHAE long non-coding RNA has the ability to change the expression of some internal control genes and then introduced *ACTB* as an appropriate reference gene for the normalization of genes expression and measured lnc-YWHAE expression level that increased $\log_2$ fold change 5 compared to the control sample (figure 3B).

Conflict of Interest

The authors have no conflicts of interest to declare.

Acknowledge

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Supplementary Information:

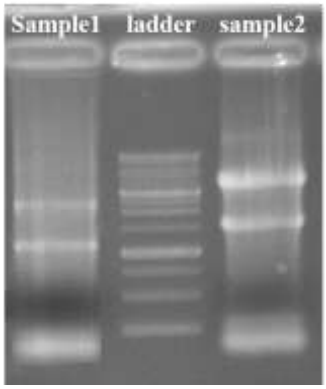

Figure S1. RNA samples on agarose gel, show differences in RNA quality.

Figure S2. PCR products on agarose, show the difference in the quality of the cDNAs.

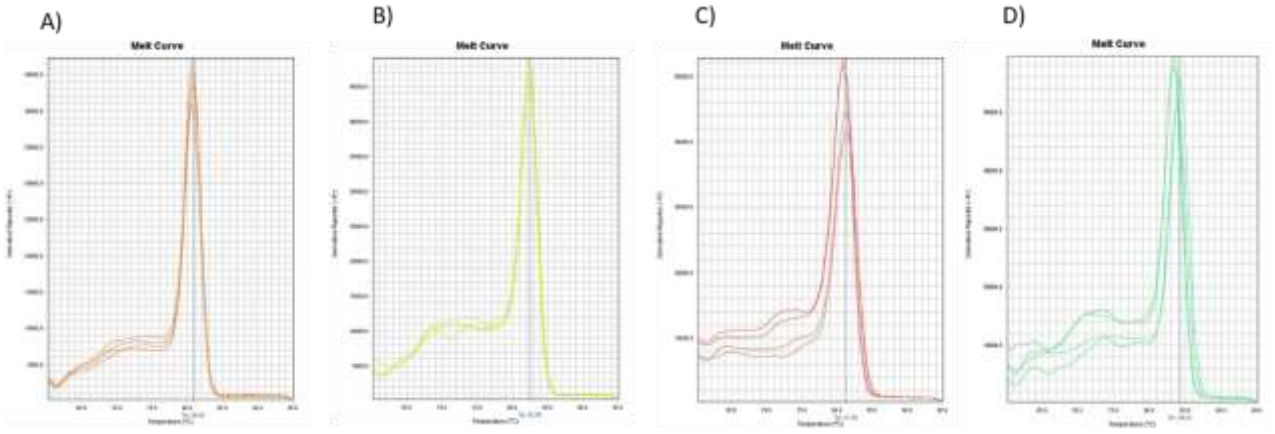

Figure S3. Melting curve diagram. Melting curve for internal control genes. A) B2M; B) GAPDH; C) ACTB; D) HPRT1.