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Research Article

Identification of Suitable Housekeeping Genes for Quantitative Gene Expression Analysis During Retinoic Acid-induced Differentiation of Embryonal Carcinoma NCCIT Cells

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Abstract

Real-time quantitative PCR (qRT-PCR) is often used as an effective experimental method for analyzing gene expression. In this method, normalization of target gene expression levels must be performed using housekeeping genes (HKGs). HKGs are used to compensate for difference between samples due to diverse quality and quality of RNAs and different reverse transcription yield. For an ideal reference gene, constant expression levels across different samples of one experiment is necessary. In the current study, expression stability of four candidate references genes including Beta actin (ACTB), glyceraldeyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine guanine phosphoribosyl transferase (HPRT1) and Beta-2-Microglobulin (β2M) following retinoic acid (RA) treatment in embryonal carcinoma NCCIT cells were evaluated. NCCIT cells were exposed to RA (10 µM) for 14 days to induce differentiation. RT-qPCR for candidate references genes was performed and normalization between untreated and RA-treated cells was performed using identical sample input amounts. Expression of OCT4, SOX2, NANOG during RA-induced differentiation was assessed by quantitative real-time PCR. RT-qPCR results indicated significant difference in expression level of GAPDH between untreated (Ct mean: 19.36667± 0.28) and RA-treated (Ct mean: 28.94± 0.18) NCCIT cells. However, transcriptional level of ACTB, HPRT and β2M remained unchanged after RA treatment. qRT-PCR analysis using ACTB, HPRT and β2M showed treatment of NCCIT cells with RA lead to significant down regulation of OCT4 (79%), NANOG (71%) and SOX2 (96%) transcript. ACTB, HPRT and β2M were recognized as valid reference genes for analysis of gene expression during RA-induced differentiation of NCCIT cells, while GAPDH was not suitable.

***Keywords*:** Housekeeping genes, NCCIT cell, Retinoic acid, Differentiation, Expression analysis

Introduction

Real-time reverse transcription quantitative PCR (qRT-PCR) has emerged as one of the most powerful tools for quantifying gene expression levels in a variety of gene expression studies. In real time PCR, it is required to normalize variations in quantity and quality of starting RNA and cDNA synthesis efficiency among different samples. For this purpose, internal control, often referred to as a housekeeping or reference gene are widely used (Nygard et al., 2007). Housekeeping genes (HKGs) are constitutive genes that are required for maintenance of basic cellular function (Richly et al., 2003). Since, HKGs play a critical role in accurate normalization of qRT-PCR data, an ideal HKGs must be expressed in a stable and non-regulated constant level across different cell types and experimental situation (Chen et al., 2006; Turabelidze et al., 2010).

Beta actin (ACTB), glyceraldeyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine guanine phosphoribosyl transferase (HPRT1) and Beta-2-Microglobulin (β2M) are among the most commonly used HKGs in qRT-PCR studies (Dheda et al., 2004; Radonić et al., 2004). However, several reports have shown that these HKGs have variable expression levels in different experimental conditions (de Kok et al., 2005; Glare et al., 2002; Selvey et al., 2001). Therefore, there is no universal HKGs having stable expression in all tissues under all experimental conditions and it is clear that suitable HKGs must be selected for particular sample sets and experimental models.

The purpose of our current investigation is to evaluate the expression stability of four candidate HKGs (ACTB, GAPDH, HPRT and β2M) during retinoic acid (RA)-induced differentiation of embryonal carcinoma (EC) NCCIT cells. According

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to our results, GAPDH showed high variability of expression between untreated and RA-treated NCCIT cells. Therefore, although GAPDH is a common reference gene in many experiments, it cannot be an optimal reference gene in this experiment. On the contrary, expression levels of ACTB, HPRT and β2M remained unchanged in NCCIT cells after RA treatment, so they can be used as a suitable reference gene for analysis of gene expression in NCCIT cells exposed to RA.

Materials and Methods

NCCIT cell culture and RA treatment

NCCIT cells (Pasteur Institute of Iran, Tehran) were cultured in RPMI-1640 medium (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, France) and 100 U/mL penicillin and streptomycin (Biowest, France) in the presence of a humidified atmosphere with 5% CO_2 at 37 °C. According to many researches, to induce differentiation, NCCIT cells were treated with 10 µM RA for 2 weeks (Jin et al., 2012; Kooistra et al., 2009; Park et al., 2014).

RNA extraction and cDNA synthesis

RNA isolation kit (DENAzist Asia, Mashhad, Iran) was used to extract total RNA from untreated and RA-treated NCCIT cells. Afterward, quality and quantity of RNA were assessed via nanodrop and agarose gel electrophoresis. To avoid DNA contamination, total RNA was treated with DNase I (Cat. No. EN0521; Thermo Fisher Scientific). For cDNA synthesis, 1 µg RNA was reverse transcribed using 200 U of M-MuLV reverse transcriptase (Cat. No. EP0441; Thermo Scientific) in presence of 5 µM oligo (dt)18 (Cat. No. MAN0013109; Thermo Scientific), 1 mM dNTPs (Cat. No. R0192; Thermo Scientific) in accordance with the manufacturer's instructions.

Quantitative Real-Time PCR

Real time PCR was performed using real-time PCR system (Analytik Jena, Jena, Germany). For each reaction, 12.5 µl of SYBR Green, 0.5 µl of forward and reverse primers (10 µM) and 1µl cDNA in total volume of 25 µl was used. Real time program for all genes **was**: 95°C for 4 min followed by 40 cycles of 95°C for 30 s, 62°C for 20s, and 72°C for 10 s. To derive melting curves, at the end of the PCR run, temperature was increased in steps of 1°C for 10 s from 61°C to 95°C. The sequence of primers and product length are described in Table 1.

Table 1. List of different PCR primers used in the study

Gene name	Sequence (5´ to 3´)	Product size (bp)
POU class 5 homeobox 1 (POU5F1) Alias symbols: OCT4	F:CCGAAAGAGAAAGCGAACCAGTAT R: CCACACTCGGACCACATCCTTC	145
Nanog homeobox (NANOG)	F: AATACCTCAGCCTCCAGCAGATG R: CTGCGTCACACCATTGCTATTCT	149
SRY-box transcription factor 2 (SOX2)	F:GGGAAATGGGAGGGGTGCAAAAGAGG R: TTGCGTGAGTGTGGATGGGATTGGTG	151
Hypoxanthine guanine phosphoribosyl transferase (HPRT)	F: TTTGTTGTAGGATATGCCCTT R: ACATTGATAATTTTACTGGCGAT	168
Beta-actin (ACTB)	F: ACCACCTTCAACTCCATCATG R: CTCCTTCTGCATCCTGTCG	120
Glyceraldehyde-3-phosphatedehydrogenase (GAPDH)	F: GACCACTTTGTCAAGCTCATTTCC R: GTGAGGGTCTCTCTCTTCCTCTTGT	151
Beta-2-Microglobulin (*β2M*)	F: CTCCGTGGCCTTAGCTGTG R: TTTGGAGTACGCTGGATAGCCT	69

Data analysis

Fold change in expression of the HKGs in RA-treated versus the untreated samples

Reverse transcription was performed with the same amount (1 µg) and optimal quality of total RNA from two samples (untreated and RA-treated NCCIT cells), so mean cycle threshold (Ct) values of two samples were used to evaluate expression stability of candidate HKG.

In order to calculate fold change in expression of the HKGs in the treated versus the untreated samples, at first mean Ct values was calculated as 2^{-Ct} and fold change is calculated as Mean 2^{-Ct} $_{\text{treated cell}}$/ Mean 2^{-Ct} $_{\text{untreated cells.}}$

Fold change in expression of pluripotency factors during RA-induced differentiation of NCCIT cells

To assess expression of OCT4, NANOG and SOX2 in RA-exposed cells in comparison with the untreated control cells, B2M, HPRT and ACTB were used as HKGs and data were analyzed by relative quantification using the comparative Ct method: Fold change = $2^{-\Delta\Delta ct}$.

$\Delta\Delta C$ = [ΔCt treated cells (Meant Ct $_{\text{target gene}}$ - Mean Ct $_{\text{reference gene}}$) _ ΔCt control cells (Meant Ct $_{\text{target gene}}$ - Mean Ct $_{\text{reference gene}}$)]

Statistical analysis

In this experiment, NCCIT cells were cultured and treated with RA in three "6 cm cell culture plate" and then differentiated cells were merge and one RNA was extracted from them. The same was done for untreated NCCIT cells. For qPCR, there are three biological repeats for each gene. Mean Ct values are shown in Supplementary table 1. T-test was used to compare expression of genes between treated and control NCCIT cells. P-value less than 0.01 was considered statistically significant.

Results and Discussion

Identification of ideal HKGs that are stably expressed under various experimental conditions and tissues of interest would provide a powerful tool for normalization of target gene expression levels in qRT-PCR. On the contrary, poor selection of HKGs can invalidate the normalization process and lead to the generation of misleading information (Chen et al., 2006; Glare et al., 2002; Haller et al., 2004). Unfortunately, it has been proved that expression of commonly used HKGs is affected with treatment or physiological state (Bustin, 2000; Janovick-Guretzky et al., 2007; Wu and Rees, 2000). Therefore, studies to find suitable HKGs with a relatively stable expression level across many different cell type and conditions have received more attention in gene expression analysis (Vandesompele et al., 2002). In the present paper we have therefore looked at the expression stability of commonly used HKGs including ACTB, HPRT, β2M and GAPDH during RA- induced differentiation of NCCIT cells.

For this purpose, normalization was performed between untreated and RA-treated samples using same amount of RNA (1 µg) in cDNA synthesis and optimal quality of RNAs were confirmed by nanodrop (260/230: 2-2.2 and 260/280: 1.8-2) and electrophoresis (supplementary Figure 1). After qRT-PCR, analysis of melting curves clearly indicated that each of the primer pairs described in Table 1 amplified a single expected product with a distinct Tm (supplementary Figure 2). The accuracy of the amplification reaction was validated by gel electrophoresis (Figure 1).

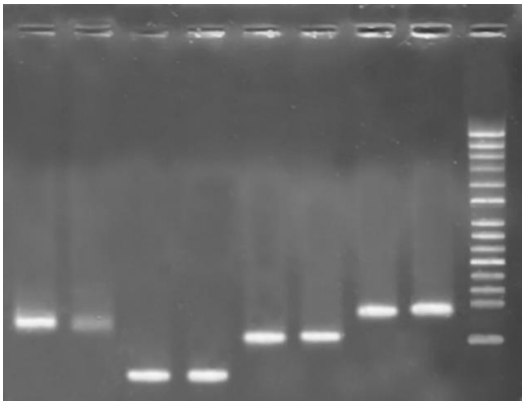

Figure 1. The accuracy of qRT-PCR was further validated by gel electrophoresis. qRT-PCR products from left to right are GAPDH: 151 bp; β2M: 69 bp; ACTB:145 b and HPRT: 168 bp genes for untreated and RA-treated NCCIT cells respectively. A molecular weight marker (100 bp ladder) is used.

Ct values of the candidate HKGs obtained by qRT-PCR in both samples (untreated and RA-treated NCCIT cells) were used to assess their expression levels and stability (supplementary Table 1). In comparison with other HKGs, the average Ct±SD (25.8±0.025) of β2M in untreated NCCIT cells was the highest, meaning it had the lowest expression level than other and the most abundantly expressed gene was *ACTB* and its average Ct±SD (17.78±0.055) was the lowest.
Using the Ct values of each experimental sample, we draw a box-plot for candidate HKGs (Figure 2). The results showed that mean Ct values of HPRT (untreated cells: 22.55± 0.12; RA-treated cells: 22.51±0.18), ACTB (untreated cells:17.785±0.05; RA-treated cells: 17.33±0.17) and β2M (untreated cells: 25.85± 0.02; RA-treated cells: 26±0.06) did not change significantly after treatment with RA. Calculating fold change in expression of the HKGs in the treated versus the untreated samples showed RA treatment changed the expression of β2M, HPRT, GAPDH by 0.86, 1.03 and 1.37 fold. Therefore, they can be regarded as reliable reference genes for expression analysis of target genes in this experiment. On the contrary, expression of GAPDH in NCCIT cells (Ct:19.36±0.28) reduced significantly (around 678-fold) after RA treatment (Ct: 28.94±0.18) and so this gene is not suitable reference gene for expression analysis of target genes after exposure to NCCIT and likely any cell lines to RA.

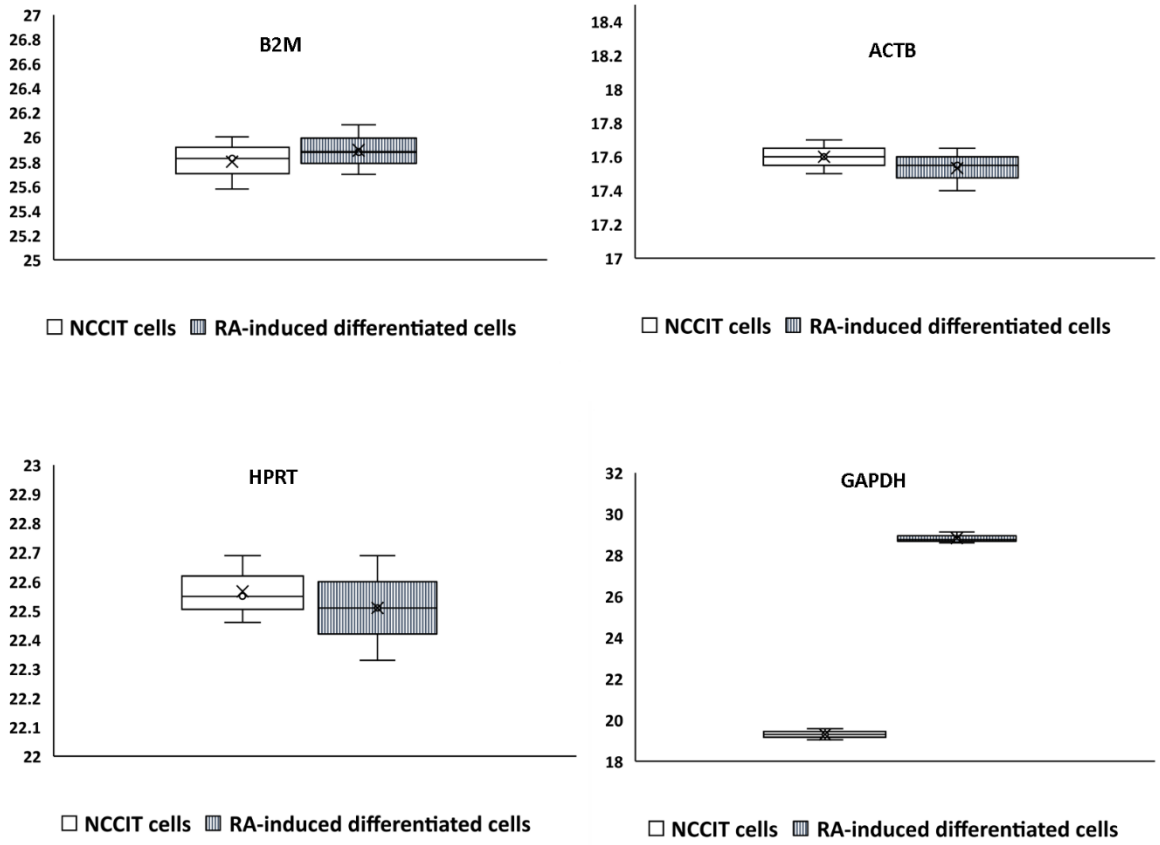

Figure 2. Box-plot based on Ct of 4 candidate HKGs in untreated and RA-treated NCCIT cells. The block diagram represents the quartile range (25th to 75th percentiles) of Ct values; the '×' in the box depicts the median of the Ct value; underline and overline are determined by the minimum and maximum values of the Ct value.

This was the first study that analyzed expression stability of some HKGs during RA-induced differentiation of pluripotent embryonal carcinoma NCCIT cells. In another study, stability of various HKGs during RA-induced differentiation of human embryonic stem (ES) cell was examined and β2M, ribosomal protein L13A and Alu repeats were found to be the most stable for this experimental set-up (Vossaert et al., 2013). According to another report, both HPRT and beta-tubulin mRNA levels varied markedly in spontaneously differentiating and growth factor-

supplemented (TGF-beta) ES cell cultures, while GAPDH expression remained relatively constant (Murphy and Polak, 2002).

RA mediated differentiation and exit from the pluripotent state in NCCIT cells were confirmed via expression analysis of important pluripotency markers including OCT4, NANOG and SOX2 using qRT-PCR. The transcription factors OCT4, SOX2, and NANOG are important factors for maintaining pluripotency and self-renewal of pluripotent cells (Chambers and Tomlinson, 2009). In many researches, down regulation of OCT4, NANOG and SOX2 were reported during differentiation of pluripotent cells and exit from pluripotency state (Rassouli et al., 2013; Soltanian and Dehghani, 2018; Soltanian et al., 2014; Soltanian et al., 2020; Stevanovic, 2003).

Since, β2M, ACTB and HPRT showed stable expression levels during RA treatment, they could be used as reference genes for expression analysis of pluripotency factors between untreated and RA-treated NCCIT cells. Results showed that after RA treatment, expression of OCT4, NANOG and SOX2 decreased to around 79 %, 71 % and 96 % of that of untreated NCCIT cells (Figure 3). Therefore, according to these results, NCCIT cells undergo differentiation by RA treatment.

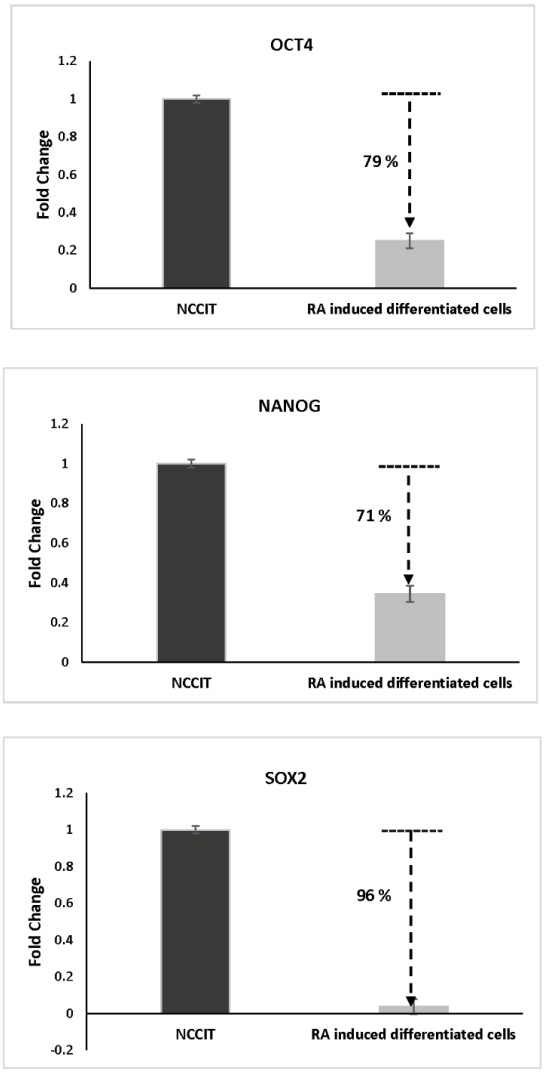

Figure 3. Quantitative RT-PCR analysis of OCT4, NANOG and SOX2 gene expression after treatment of NCCIT with RA. NCCIT cells were treated with RA (10 μM) for 14 days and expression of OCT4, NANOG and SOX2 were measured and compared to untreated cells as control by real time PCR. The graph represents the mean data±SD (error bar) of at least three independent experiments. *$p<0.01$ against control.

In conclusion, selection of a suitable HKG is essential to normalize real-time PCR data. However, the choice of HKGs should be tailored to the nature of the study. For example, although

B2M, ACTB, HPRT and GAPDH are widely used and accepted as reference genes for analysis of gene expression, according to some researches, their expression varies in different situation. Therefore, expression level stability of HKGs must be confirmed in each study (Guénin et al., 2009; Zhang et al., 2005). Furthermore, it has been strongly proposed that more than one stable expressed reference gene should be used to avoid misinterpretation of gene expression data (Hamalainen et al., 2001; Zhong and Simons, 1999).

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Conflict of Interest
The authors declare that they have no conflict of interest

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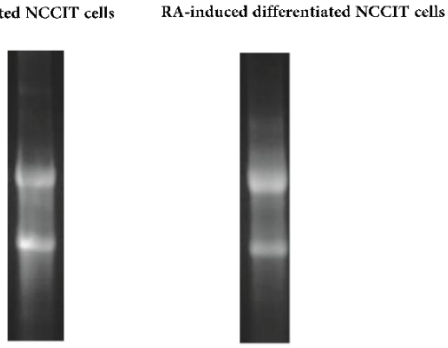
Supplementary Materials:

A



B

Sample	Concentration	260/280	260/230
Undifferentiated NCCIT cells	1000 µg/ml	2.1	1.8
RA- induced differentiated cells	1000 µg/ml	2.2	1.9

Figure S1. Agarose gel electrophoresis of total RNA isolated from undifferentiated and RA- induced differentiated NCCIT cells. A) Intact total RNAs have sharp 28S and 18S rRNA bands. B) Nanodrop analysis of RNA samples isolated from undifferentiated and RA- induced differentiated NCCIT cells.

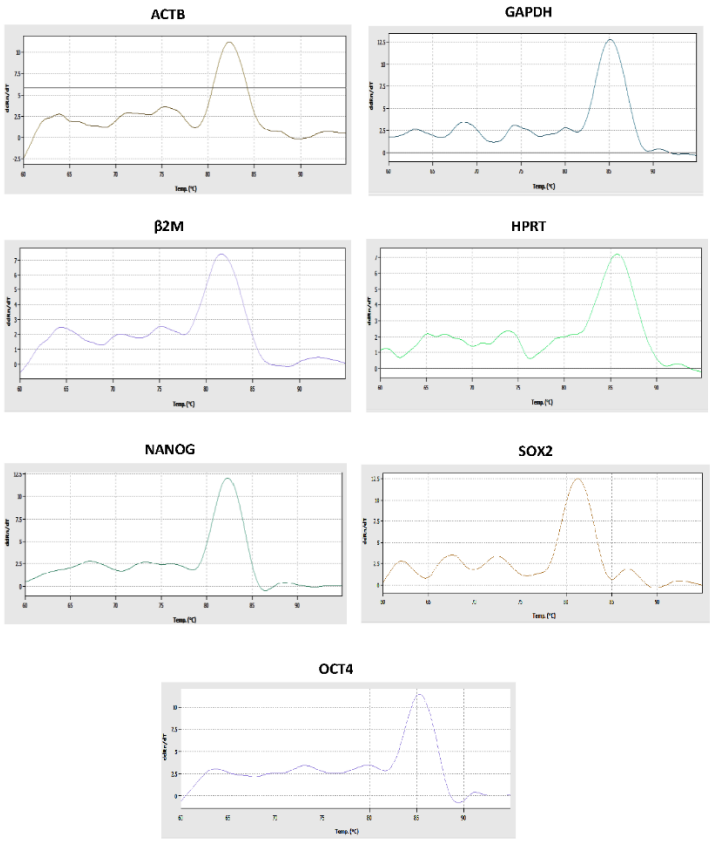


Figure S2. Melt-curve analysis of products from SYBR Green assay for each primer sets.

Table S1. The man Ct value of three biological repeats

Gene Name	β2M	HPRT	GAPDH	ACTB
Ctvalues	25.88	22.69	19.58	17.84
(NCCIT	25.83	22.5	19.04	17.73
untreated	25.85	22.46	19.48	17.785
cells)				
Ctvalues	25.99	22.69	29.12	17.16
(RA-	26.12	22.33	28.76	17.51
treated	26.09	22.51	28.94	17.335
NCCIT				
cells)				