A Comparison of GAPDH and ACTB As Internal Control for Gene Expression Studies in Different Cancer Cell Lines

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ABSTRACT

Housekeeping genes are used as internal controls to normalize the expression of target genes in gene expression studies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Actin beta (ACTB) are frequently preferred as housekeeping genes in gene expression studies. Due to the general alterations in the gene expression pattern in cancer cases, the selection of the appropriate housekeeping genes for these studies are challenging. In this study, we aimed to analyze the expression of the well-known housekeeping genes GAPDH and ACTB in 6 different cancer cell lines. Furthermore, the relative gene expression of the selected target gene (Tissue inhibitor of metalloproteinases 2-TIMP2) was normalized separately using GAPDH and ACTB and the obtained results were compared with each other. Finally, the stability of GAPDH and ACTB was analyzed using the in-silico tool, Bestkeeper. As a result of the study, it is found that the expression of GAPDH and ACTB were significantly different in the Jurkat ($p< 0.01$), MOLT4 ($p< 0.05$), REH ($p< 0.001$) and HT29 ($p< 0.001$) cell lines. Based on this finding, significantly different relative target gene expression values were obtained in different cell lines depending on whether the selected housekeeping gene was GAPDH or ACTB. In addition, GAPDH was found to show less variation among the samples used in all cell lines and more stability based on Bestkeeper analysis. These results support that the appropriate housekeeping gene selection, especially in cancer cell lines, may be an effective factor in obtaining accurate results for the studies in the field provide guidance to researchers.

Keywords: Cancer cell lines, Gene expression, Housekeeping genes, GAPDH, ACTB

INTRODUCTION

Today, it is quite possible to obtain much more detailed information about the cell and its metabolism with the developing molecular biology techniques. Gene expression, which is the first step in the process of producing proteins that are the cornerstone of cellular functions, is very significant in this respect. In measuring the expression of a particular gene, a different gene constitutively expressed at a certain level in the cell is used as an internal control.[1-4] Although these genes are called with different names (internal control, reference gene, housekeeping gene, etc.), in this study it has been preferred to address them as housekeeping genes (HKG). The number of genes used as HKG in the human genome varies as our knowledge of structure and function of the cell increases. According to the Housekeeping and Reference Transcript Atlas (HRT Atlas v1.0, www.housekeeping.unicamp.br), which was generated by mining massive human and RNA-seq data sets in a recent study, 2176 housekeeping genes are listed.[5]

Reverse transcription quantitative polymerase chain reaction (RT-qPCR), which is a simple but effective method, is preferred to analyze the gene expression of the target genes in the desired tissue or cell lines.[6] Additionally, RT-qPCR analyses are very essential for the characterization of the cancer cells.[7,8]

However, it is necessary to normalize the results using an HKG when performing the analysis. HKGs are perfect controls since they are not co-regulated with target genes or not affected by environmental and experimental conditions.[9] For this reason, the selection of a proper HKG during the study design is one of the most critical and result-affecting stages of a study.[10]

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Actin beta (ACTB) are frequently preferred HKGs in both human and cell culture studies, especially for cancer research.[10] This may be explained by the fact that mRNA expression levels of these genes were found to be high and constant in many different cells and tissues.[2,11,12] However, there may be high gene expressions at the whole transcriptome level in different cancer types. Due to the nature of cancer these differentiated expressions affect the cells in different ways. Many studies indicate that known reference genes show expression changes in different cancer types.[13-15] Thus, the selection of the gene that represents intracellular control in each cancer types requires a more specialized effort.[11]

To determine how carcinogenesis is driven by genetic changes, genetic studies in oncology have concentrated on quantifying the molecular alterations between cancer and normal cells.[10] Within the scope of this kind of study design, cell lines are very valuable and versatile tools as they allow researchers to work individually for each cancer type. In other words, they allow many studies to be carried out simultaneously without human samples.[16-18] Many challenges such as the progression of cancer disease, the impossibility and inconvenience of continuous tissue sampling (on the different stages of the disease) from the patient can be overcome with the use of cell lines. Considering the genetic heterogeneity of cancer tissue, cell lines are not always 100% representative of the relevant cancer, but they are very significant in the investigation of disease pathogenesis and the accumulation of scientific knowledge.[19,20]

The aim of this study was to determine the differences in the expression of GAPDH and ACTB genes (Table 1) in cell lines representing different cancer types (Jurkat (T cell leukaemia), K562 (chronic myelogenous leukaemia), MOLT4 (acute lymphoblastic leukaemia - T lymphoblast), REH (acute lymphocytic leukaemia - B cell precursor leukaemia), HT29 (colon adenocarcinoma) and MDA-MB-231 (breast adenocarcinoma) by using RT-qPCR. It was also aimed to determine how a potential expression difference between GAPDH and ACTB would be reflected in the result of normalization of a target gene. Within the scope of the study, tissue inhibitor of matrix metalloproteinase 2 (TIMP2) gene was selected as the target gene to investigate differentiated gene expression. Invasion and metastasis, both of the most characteristic features of cancer, are closely related to remodeling of the extracellular matrix (ECM).[21] Major regulators of ECM are matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs).[22] The TIMP2 gene was included in the study in order to show the difference in expression of any selected target gene. Furthermore, TIMP2 is known to have altered expression in different cancer types shown in the literature.[21,23-25] Due to the fact that TIMP2 was considered to have a high power to represent gene expression changes in cancer cells, it was included in the study. It is also expected that the results of the study would assist researchers to decide the selection of HKGs in future studies which are focus on the specific cancer cell lines.

Table 1. Information about candidate housekeeping genes

Gene Name	Symbol	Primer Sequences (5' to 3')	Product Size
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Forward: AGTCAACGGATTTGGTCGTATTG Reverse: TGGGTGGAATCATATTGGAACAT	138
Actin beta	ACTB	Forward:CACCTTCTACAATGAGCTGCGT Reverse: AGGCGTACAGGGATAGCACAG	170

GAPDH= Glyceraldehyde-3-phosphate dehydrogenase, ACTB= Actin beta

Table 2. Mean Cq and SD values of GAPDH and ACTB genes

Gene Name	Jurkat (Mean±SD)	K562 (Mean±SD)	MOLT4 (Mean±SD)	REH (Mean±SD)	HT29 (Mean±SD)	MDA (Mean±SD)
GAPDH	28.96±0.078	31.63±0.50	36.11±1.0	32.21±0.15	27.23±0.14	29.28±0.39
ACTB	30.10±0.37	32.05±0.21	34.97±0.54	35.43±0.45	28.78±0.20	29.69±0.31

Values were expressed as the mean ± standard deviation (SD). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, ACTB: Actin beta, Cq: Cycle of quantification, Jurkat:T cell leukaemia, K562: Chronic myelogenous leukaemia, MOLT4: Acute lymphoblastic leukaemia - T lymphoblast, REH: Acute lymphocytic leukaemia - B cell precursor leukaemia, HT29: Colon adenocarcinoma, MDA: Breast adenocarcinoma

MATERIALS AND METHODS

Cell Culture

In this study, the chosen suspended and adherent cell lines were cultured under appropriate conditions. Jurkat, K562, MOLT4, REH, as suspended cell lines were cultured in RPMI 1640 plus containing 1% Penicillin-Streptomycin and 10% heat-inactivated fetal calf serum (FCS). HT29, MDA-MB-231 as adherent cell lines were cultured in DMEM containing 1% Penicillin-Streptomycin and 10% heat-inactivated fetal calf serum (FCS). Culturing conditions were containing 5% CO_2 at 37°C. All cell lines were cultured at a density of $2.5x10^5$/ml in 5 ml medium. Cells were cultured for 5 days. At the end of the culturing period, RNA isolation was performed for the gene expression study. All cell lines were kindly provided by Department of Genetics and Bioengineering in Istanbul Bilgi University.

RNA Isolation and cDNA Synthesis

RNeasy Mini Kit (Qiagen, Cat. No: 74104) was used for RNA isolation of cultured cells. For the isolation, the protocol recommended by the kit provider is followed. The quality and purity of the isolated total RNA were measured with NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Total RNA from each cell line was reverse transcribed into cDNA with iScript cDNA Synthesis Kit (Bio-Rad, Cat. No: 1708890) by following the manufacturers protocol for further analyses.

Quantification with RT-qPCR

Transcript analysis of GAPDH and ACTB was conducted by the quantitative reverse transcription polymerase chain reaction (RT-qPCR) using the LightCycler 96 system (Roche). For each reaction, cDNA equivalent to 20 ng/μl RNA was used. The reaction mixture was prepared by using 10 μl master mix (Light Cycler 480 SYBR Green I Master, Roche, Cat. No: 04707516001), 3 μl primer mix (2.5 μM for each), 5 μl water (nuclease free) and 2 μl cDNA (10 ng/μl). PCR cycling conditions are as follows. 1 cycle preincubation 95^0C for 300s, 45 cycle of 3 step amplification; 95^0C for 15s, 60^0C for 20s, 72^0C for 20s, and 1 cycle of cooling 40^0C for 30s. All PCRs set up for each sample were run in triplicate to ensure reliability and accuracy of the results.

Statistical Analysis

All statistical analyses are performed using GraphPad Prism version 9.3.1 (Dotmatics). Numerical data are presented as mean and standard deviation (SD). Shapiro-Wilk test is used to determine whether the data were normally distributed. Student's t test is used for continuous variables which fit normal distribution in the study group. To perform the multiple comparisons for the datasets containing 3 or more groups, analysis of variance (ANOVA) followed by a post hoc test (Tukey) is used. For the gene expression stability analysis, Bestkeeper (10, 26) software is used. For all statistical analyses, p-values $p \leq 0.05$ are considered statistically significant.

RESULTS

Expression Profiles of ACTB and GAPDH in Selected Cell Lines

The mRNA expression level differences of GAPDH and ACTB were calculated by mean cycle quantification (Cq) analysis. The Cq values for both GAPDH and ACTB obtained from all indi-

Table 3. Statistical significance levels of GAPDH gene expression difference in between cell lines

	K562	MOLT4	REH	HT29	MDA
Jurkat	0.00***	0.00****	0.00**	0.21	0.99
K562		0.00****	0.71	0.00****	0.00***
MOLT4			0.00***	0.00****	0.00****
REH				0.00****	0.00**
HT29					0.09

*Analysis method: one-way ANOVA post-hoc Tukey test. n =3. Values were expressed as p value. P ≤ 0.05 is considered as statistically significant. *p ≤ 0.05 , ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001. GAPDH; Glyceraldehyde-3-phosphate dehydrogenase, Jurkat:T cell leukaemia, K562: Chronic myelogenous leukaemia, MOLT4: Acute lymphoblastic leukaemia - T lymphoblast, REH: Acute lymphocytic leukaemia - B cell precursor leukaemia, HT29: Colon adenocarcinoma, MDA: Breast adenocarcinoma*

vidual cell lines are given in Table 2. Due to the nature of the method, the Cq value is inversely proportional to the initial amount of nucleic acid (mRNA). In other words, a lower Cq value indicates higher expression, while a higher Cq value, in contrast, indicates a relatively low amount of mRNA and obviously lower expression. We would like to emphasize that the terms 'lower' and 'higher' are used here in a descriptive sense. Statistical significance is not meant at this point.

To answer the question whether the gene expression of each HKG (GAPDH and ACTB) showed a difference through the cell lines or not, mRNA expressions of the individual HKGs were analyzed separately in the studied cell lines. The one-way ANOVA and post-hoc Tukey tests are used to determine expression differences in between cell line groups. The obtained results are given as cross tables in Table 3 and Table 4. p≤ 0.05 is considered as statistically significant. For decimal point consistency in the data presented, all p values are given as two digits after the comma. In order to express the statistical significance of smaller values, different numbers of asterisks are used.

Expression Differences and Stability of GAPDH and ACTB in the Same Cell Line

In the concept of this study, the expressions of GAPDH and ACTB were examined in order to observe whether there was a significant difference between them in the same cell line. Therefore, GAPDH and ACTB expression values were compared in cell line groups by using Student's t test. The obtained results are given in Figure 1. p≤ 0.05 is considered as statistically significant. The stability values of the HKGs obtained from the in-slico tool, Bestkeeper (10, 26), are also given in Figure 2.

Determination of a Relative Gene Expression of the Target Gene by Using GAPDH and ACTB Separately

Since there was a significant difference in Cq values of GAPDH and ACTB in Jurkat, MOLT4, REH and HT29 cell lines, a target gene (TIMP2) was studied to show that there would be a statistically significant difference in the relative gene expression results. Cq values were analyzed by Delta Delta Cq (ΔΔCq) method.[27] In Jurkat, MOLT4,

Table 4. Statistical significance levels of ACTB gene expression difference in cell lines

	K562	MOLT4	REH	HT29	MDA
Jurkat	0.00***	0.00****	0.00****	0.00**	0.74
K562		0.00****	0.00****	0.00****	0.00****
MOLT4			0.66	0.00****	0.00****
REH				0.00****	0.00****
HT29					0.07

*Analysis method: one-way ANOVA post-hoc Tukey test. n =3. Values were expressed as p value. p ≤ 0.05 is considered as statistically significant. * p ≤ 0.05 , ** p ≤ 0.01, *** p ≤ 0.00 1, **** p ≤ 0.0001. ACTB: Actin beta, Jurkat:T cell leukaemia, K562: Chronic myelogenous leukaemia, MOLT4: Acute lymphoblastic leukaemia - T lymphoblast, REH: Acute lymphocytic leukaemia - B cell precursor leukaemia, HT29: Colon adenocarcinoma, MDA: Breast adenocarcinoma*

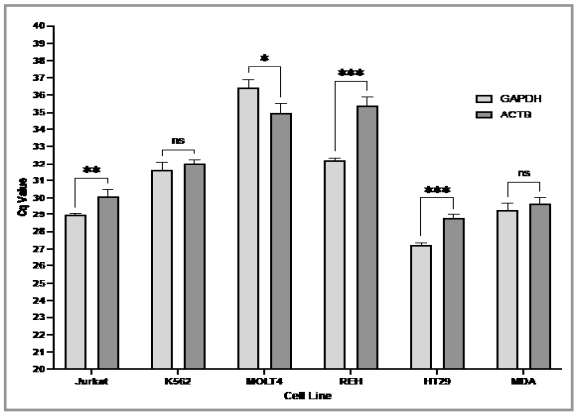

Figure 1. Expression differences of GAPDH and ACTB among the human cell lines. p≤ 0.05 is considered as statistically significant. *p≤ 0.05 , **p≤ 0.01, ***p≤ 0.001, ns: not significant.

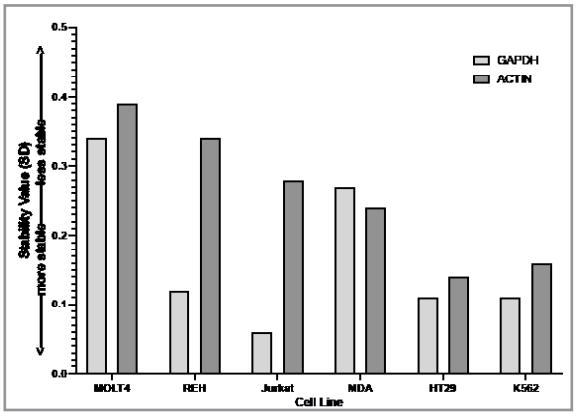

Figure 2. Expression stability values of the candidate reference genes for MOLT4, REH, Jurkat, MDA, HT29 and K562 cell lines by Bestkeeper.

REH and HT29 cell lines, the relative expression level of the target gene (TIMP2) was significantly different from the control group when GAPDH was used as an internal control. In none of the cases where ACTB was used as internal control, no difference was observed in the expression of the target gene compared to the control group. The results are given in Table 5.

DISCUSSION

As any function within the cell occurs as a projection of gene expression, it is important to be able to quantify it. HKGs are used for normalizing the gene expression measurements. The studies show that there is no universal HKG that can be used for all studies. It is because, depending on the experimental conditions like hypoxia and serum deprivation[28,29], the stability of the generally used HKGs can be modified.[30,31] Especially in the cancer cells, expression patterns and the stability of housekeeping genes may vary.[32] Therefore, choosing an appropriate HKG for the target disease becomes more critical. In this study, we primarily aimed to investigate the expression of the generally preferred GAPDH and ACTB housekeeping genes in different cancer cell lines. We also aimed to investigate whether a significant difference would be obtained when they were used in target gene normalization. In this way, it is aimed to emphasize the importance of HKG selection and underline the requirement of a preliminary study during study design. Jurkat (T cell leukaemia), K562 (chronic myelogenous leukaemia), MOLT4 (acute lymphoblastic leukaemia - T lymphoblast), REH (acute lymphocytic leukaemia - B cell precursor leukae-

Table 5. Relative Gene Expression of TIMP2 gene which is normalized by GAPDH and ACTB separately in different cell lines

	HKG used for normalization	
	GAPDH	**ACTB**
Jurkat	0.93	0.16
MOLT4	0.03*	0.11
REH	0.03*	0.08
HT29	0.00**	0.25

*Values were expressed as p value. P ≤ 0.05 is considered as statistically significant. *p≤ 0.05 , ** p≤ 0.01*
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, ACTB: Actin beta, TIMP2: Tissue inhibitor of metalloproteinases 2, Jurkat:T cell leukaemia, MOLT4: Acute lymphoblastic leukaemia - T lymphoblast, REH: Acute lymphocytic leukaemia - B cell precursor leukaemia, HT29: Colon adenocarcinoma.

mia), HT29 (colon adenocarcinoma) and MDA-MB-231 (breast adenocarcinoma) cell lines are used to perform the study.

In the scope of this study, the expressions of GAPDH and ACTB were analyzed individually in cell lines. As a result, it was determined that both GAPDH and ACTB showed different expression levels in each cell line (Table 2). In many studies, it has been reported that housekeeping genes can show variable gene expression values in different cancer types.[13-15] In this point of view, the results obtained in our study are consistent with the literature. In addition, when the individual expressions of GAPDH and ACTB were compared among cell lines, the differences obtained were statistically significant (Table 3 and Table 4). Multiple comparison analysis showed that the same housekeeping gene may be expressed at different levels in different cell lines. In the light of the literature[13-15,32], it can be expected that a certain gene is expressed differently in different cancer types. However, we think it may be noteworthy that statistically significant differences in the GAPDH and ACTB genes were obtained even in Jurkat, K562, MOLT4, and REH cell lines which are representing different types of leukaemia (Table 3 and Table 4).

Additionally, in the results of this study, it was also found that there was a significant difference between GAPDH and ACTB expression in Jurkat ($p< 0.01$), MOLT4 ($p< 0.05$), REH ($p< 0.001$) and HT29 ($p< 0.001$) cell lines (Figure 1). This is also consistent with the Cq values given in Table II. To explain functionally, GAPDH is involved in glycolysis steps. It has also been reported to be involved in different cellular functions such as DNA replication and repair process, nuclear RNA molecule export, exocytosis and cytoskeleton dynamics.[6] ACTB is a cytoskeletal element and it is essential for the maintenance of cell morphology.[10,33] Considering the dramatic effects of cancer cells on glucose metabolism[34] and cell morphology[35], changes in GAPDH and ACTB expression can be seen in different cell lines.[36] However, the expression changes of GAPDH and ACTB in the same cell line are remarkable (Figure I). That is because the expression measurements of target genes, which are the main focus of the studies, can also change accordingly. Based on this fact, it can be interpreted that every HKG may not be suitable for every single cell type. To validate this approach, a target gene (TIMP2) is included in the study and it is normalized with GAPDH and ACTB separately. When GAPDH is used as the HKG for normalization, statistically significant changes in the relative gene expression value of the target gene (TIMP2) were observed in MOLT4 ($p= 0.03$), REH ($p= 0.03$) and HT29 ($p< 0.01$) cell lines (Table 5).

When the ACTB gene was used for normalization, no significant difference in relative gene expression of the target gene (TIMP2) was observed in any of the cell lines. Although the use of a single housekeeping gene in gene expression studies is not uncommon[37], this strategy has also been reported to cause errors.[10,38,39] With this analysis, rather than proving that GAPDH or ACTB is more preferable, it is intended to remind researchers that their study results may vary according to the HKG they used.

Bestkeeper was used to determine the stability of HKGs in-slico. A lower standard deviation points to a more stable expression for BestKeeper.[10,26] GAPDH was detected to be more stable with lower standard deviation in cell lines except MDA (Figure 2).

Conclusion

As a result of our study, we have revealed that GAPDH and ACTB are differentially expressed in different cancer cell lines (Jurkat, K562, MOLT4, REH, HT29 and MDA). Due to the increased gene expressions in cancer cell lines, the importance of appropriate housekeeping gene selection in designed studies is obvious. Based on our results, we can conclude that there is a more stable expression of GAPDH in the cell lines we used. Although the expression of ACTB is more variable among samples, this result does not imply that it should be completely excluded.

The main findings obtained from the study underline that choosing the appropriate housekeeping gene is closely related to the accuracy of the data to be obtained as a result of the study while using different cancer cell lines. Therefore, to ensure the accuracy of the studies, it can be said that performing preliminary studies (experimental/in-slico on

specific tissue or cell line) is a very useful approach to choose an appropriate housekeeping gene.

Limitations of the study: Our study has certain limitation. We can refer it as follow. geNorm[39] and Normfinder[40], which are in slico tools developed to measure the stability of HKGs, could not be used in our study. It is because these tools can calculate the stability of the genes in case of 3 or more HKGs due to the algorithm they use. This situation will definitely be taken into consideration for future studies.

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