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Reliable Reference Gene for Normalization of RT-qPCR Data in Human Cancer Cell Lines Subjected to Gene Knockdown

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Abstract: Quantitative real-time Polymerase Chain Reaction (RT-qPCR) has become a valuable molecular technique in biomedical research. The selection of suitable endogenous reference genes is necessary for normalization of target gene expression in RT-qPCR experiments. The aim of this study was to determine the suitability of each *18S* rRNA and *ACTB* as internal control genes for normalization of RT-qPCR data in some human cell lines transfected with small interfering RNA (siRNA). Four cancer cell lines including MCF-7, T47D, MDA-MB-231 and Hela cells along with HEK293 representing an embryonic cell line were depleted of E2F6 using siRNA specific for E2F6 compared to negative control cells, which were transfected with siRNA not specific for any gene. Using RT-qPCR, Ct (threshold cycle) values of *18S* and *ACTB* were determined in transfected cells and compared with control cells. In the selection of the above cell lines, *18S* was identified as the most stably expressed reference gene than *ACTB* in gene knockdown experiments.

Keywords: Reference gene, RT-qPCR, human cancer cell lines, gene knockdown, *18S*, *ACTB*

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Introduction

Quantitative real-time polymerase chain reaction (RT-qPCR) is simple, highly sensitive, quantitative, reproducible and robust technique for investigating gene expression changes and determining transcript abundance (1). The selection of suitable reference genes is critical for target gene normalization in RT-qPCR experiments. Inaccurate normalization can lead to incorrect quantification and misinterpretation of qPCR findings (2). Several mathematical approaches have been developed to select appropriate reference gene, these approaches include: geNorm (2), NormFinder (3), Best keeper (4) and comparative delta Ct (5). Ct is the number of cycles required for the fluorescence signal to reach a specific threshold and is inversely correlated to the complementary DNA (cDNA) inputamount(6). Conventionally applied reference genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-2-microglobulin (B2M) have been demonstrated to be controlled in different situations in various tissues (7). The expression of the above genes is susceptible to hormones, and may be modified during cellular processes, for instance differentiation and cancer progression (8, 9). B2M have been reported to be affected by factors found in brain cortices in alcohol addictive persons (10). In many studies, *GAPDH*

was among the least stably expressed reference gene (11, 12, 7). *GAPDH* expression has been found to be altered in various disease stages and human cancers (13, 14, 15), and certain experimental conditions could affect its expression (11). Therefore, it is important to use suitable reference genes for each experimental set-up particularly for studies exploring subtle differences in gene expression. Such studies are susceptible to minor fluctuations of the reference genes that might result in false conclusions of the target gene expression. It is agreed to use more than one reference gene to decrease the sensitivity to degraded RNA and to variable quantities of input RNA (16). However, because of financial reasons and/or limited material, a single reference gene is usually used for RT-qPCR normalization. In a previous study (17), the expression of five reference genes including: heat shock protein 90kDa alpha family class B number 1 (*HSPCB*), ribosomal protein S13 (*RPS13*), *18S* (rRNA), small nuclear RNA (snRNA*; USB1*), and β-actin (*ACTB*) was investigated in a number of breast cancer cell lines. The cell lines included: MCF-7, T-47D, MDA-MB-231 and MDA-MB-468 compared with the normal breast cDNA. Interestingly, both *18S* and *ACTB* were the most stably expressed reference genes in both normal and cancer cells without any treatment. Therefore, the aim of this study was to further identify and investigate the expression stability of *18S* and *ACTB* genes in cells transfected with small interfering RNA(siRNA). Inhibition of gene expression by RNA interference (RNAi) is a transient phenomenon (18). RNAi is a highly evolutionally preserved and specific method that causes post-transcriptional gene silencing, when introduced into a cell, resulting in sequence-specific degradation of homologous mRNA sequences (19, 20). RNAi in mammalian cells is mediated by double stranded RNAs (dsRNAs) of 21-23 nucleotides long (21). si-E2F6 oligonucleotide was chosen as a model to disrupt specific gene expression in order to detect whether this treatment affects the expression stability of *18S* and *ACTB* or not.

Materials and methods

Cell culture

All cell lines used in this study were obtained from Sigma-Aldrich. The cell lines included: two estrogen receptor positive (ER+) cell lines MCF-7 and T47D, two triple-negative cells MDA-MB-231 and MDA-MB-468, immortalized breast epithelial cell line MCF-10A, HeLa and HEK293. All of those cells, except MCF-10A, were grown in Dulbecco's modified eagles medium (DMEM; Lonza) containing 4.5 g/L glucose with L-glutamine, and supplemented with 10% fetal calf serum (FCS; Seralab) and 1x non-essential amino acids (NEAAs; Bio Whittaker). The above cell lines were preserved with freezing medium, which was prepared by adding 10% DMSO to complete DMEM. Concerning MCF-10A cells, they were grown in DMEM containing 4.5 g/L glucose with L-glutamine with addition of 1x NEAAs, 5% horse serum (Invitrogen), insulin 10 µg/ml (Sigma-Aldrich), cholera toxin 0.1 µg/ml (Calbiochem), epidermal growth factor 10 µg/ml (EGF; Sigma-Aldrich) and hydrocortisone 50 µM (Sigma-Aldrich). Freezing medium used

for MCF-10A composed of the media used to grow them with 10% DMSO.

SiRNA transfection

All siRNAs used in this study was purchased from Eurofins, Germany. Gene depletion has been achieved by applying siRNA specific for *E2F6* including: si-E2F6#1 sense 5-AAGGAUUGUGCUCAGCAGCUG-3, si-E2F6#2 sense 5-AGUUAAAGCUCCAGCAGAA-3 and si-E2F6#3 sense 5-CUUAAGAAGUGCUCAAUAA-3. As a control, non-specific siRNA (scrambled) was used to transfect the cells. Dharma FECT®#4 (Thermo Scientific) was the transfection reagent. Reverse transfection protocol was optimized for each cell line, Additionally, different cell numbers were transfected onto 6-well plate format or different concentrations of scrambled siRNA control were used.

Briefly, 20 µM siRNA made in 1x siRNA buffer provided was mixed by pipetting with SFM (serum free medium) in an Eppendorf tube, and was left for 5 min at room temperature. Synchronously, the appropriate amount of Dharmafect® #4 was added to SFM in an Eppendorf tube and left for the same time as before. Afterwards, siRNA-SFM was mixed by pipetting carefully up and down with the Dharmafect-SFM and incubated at room temperature for a further 25 min. During that time, healthy cell lines with about 80% confluence and grown overnight in a medium without antibiotic were trypsinized, counted and diluted in an antibiotic-free complete medium. Appropriate amount of the mixture (siRNA-Dharmafect-SFM) was then pipetted to the bottom of the well. Afterwards, cells were added at the optimized density into each well and mixed with the previously added complex of siRNA-Dharmafect-SFM. Plates were incubated at 37°C with 5% CO_2 and humidity for 24 or 48 h.

RNA Extraction

To analyze *E2F6, 18S* and *ACTB* gene expression at the mRNA level in the cell lines under study, after harvesting the cell pellets, RNA was extracted using RNeasy® Mini Kit (Qiagen). Manufacturer's instructions were applied to obtain total RNA. RNA was eluted in 50 µl RNase free water and RNA concentration measured using NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA was converted immediately to cDNA, or kept at -80˚C till use.

Reverse Transcription

Following extraction, total RNA was converted to cDNA using a High Capacity RNA-to-cDNA kit (AB Applied Biosystems) in a total volume of 20 µl. The recommendations of the manufacturer were followed. The cDNA was kept at -20˚C until further use.

Primers for RT-qPCR

Oligonucleotides designed for *E2F6*, *18S* and *ACTB* were bought from Eurofins, Germany. *E2F6*, *18S* and *ACTB* primer sequences were *E2F6* Fwd: 5-TTCCAGCTCCCAGAGAAGA C-3, *E2F6* Rev: 5-TTACTGGTCTGA CCCTGCTCCA-3, 18S Fwd: 5- AGAA ACGGCTACCACATCCA-3, *18S* Rev: 5-CACCAGACTTGCCCTCCA-3,

ACTB Fwd: 5- CAGCCATGTACGTT GCTATCCAGG-3 and *ACTB* Rev: 5-AGGTCCAGACGCAGGATGGCATG-3. Each primer set was checked for its specificity to its target gene with no homology to other sequences using human genome browser (http://genome .ucsc.edu). The primers were used in RT-qPCR to detect the expression of those genes in the above cell lines. *18S* and *ACTB* primers were used in 5 µM concentration, whereas primers specific for *E2F6* were diluted to 10 µM concentration using deionized water prior to use and stored at -20°C.

RT-qPCR

RT-qPCR was conducted using a Corbett Robotics Rotor-Gene™ 6000 (Qiagen) to study the expression of *E2F6, 18S* and *ACTB*. Each reaction consisted of 20 µl of the following: 10 µl of 2× SensiMix containing a mixture of: buffer, dNTP, HiRox, SYBR Green and modified Taq polymerase. Additionally, the reaction included: 3 µl of deionized sterile water, 2 µl primers (concentration of 10µM) with 5 µl cDNA as a template. Reactions were carried out in quadrate or triplicate technical repeats. Analysis of melt curve was applied to check for presence of primer dimmers as well as amplification of a single product. The cycling conditions were 95°C for 10 minutes for Taq polymerase activation followed by 40 cycles of denaturing at 95°C for 15 s, annealing at 58°C for 15 s and finally 30 s at 72°C for extension. PCR product quantity gained is proportional to the fluorescence signal. Using Rotor-Gene 6000 software, Ct value was determined for each cDNA sample in every reaction. Each experiment included a no-template control.

Statistical Analysis

SPSS 22.0 software (IBM) for Windows was used to analyse the data. Comparisons among different cancer cell lines were analysed using non-parametric Mann-Whitney U test. The differences between Ct values of each sample were considered significant at confidence levels larger than 95% ($P < 0.05$).

Results

Quality and quantity of RNA samples

The concentration of RNA extracted from the tested cell lines was measured using NanoDrop spectrophotometer, and 1 µg of total RNA was then converted to cDNA.

qPCR efficiency in un-treated cells

The RT-qPCR efficiency of reference or target genes was determined by 10-fold dilution series of 1/10, 1/100, 1/1000, 1/10,000 and 1/100,000 conducted on cDNA extracted from MDA-MB-468 cells. These cDNA dilutions were amplified by primers specific for *18S* and *ACTB* using RT-qPCR, and the raw data (Ct values) were analyzed by RT-qPCR software (Figure 1: A). Using melt-curve analysis of RT-qPCR amplicons, no primer dimmers nor amplification of contaminating genomic DNA (gDNA) was observed (Figure 1: B), indicating that the primers used were highly specific for reference genes. By importing data to Microsoft Excel or Prism software, standard curve was plotted to show the relationship between Ct value (y-axis, linear values) *versus* cDNA concentration (x-axis,

logarithmic values). The relationship was calculated by linear regression to find a slope and correlation coefficient (R^2). The amplification efficiencies for *18S* and *ACTB* were determined using the formula E= $10^{(-1/slope)}$ (22). Using the equation of the curve, as shown for *ACTB* (y= -3.5267), the slope equals to -3.5267. Applying the efficiency equation, the efficiency of *ACTB*=$10^{(-1/-3.5267)}$, E= 1.9 (also equals to 92%). Whereas for *18S*, the slope=-2.5093 and E= 2.5 (equals to 150%). According to this finding, it looks that the amplification efficiency of *ACTB* is better than *18S*, in which an E value of more than 100 is not good. It is clear from (Figure 1) that qPCR reaction of *18S* is saturated at high concentration, and this saturation influences the efficiency of particularly *18S*. Therefore, when deleting the highest concentration (0.1) from the standard curve, higher amplification efficiency was obtained for *18S* (E= 2.2), and the efficiency of *ACTB* was (E= 1.9) (Figure 1: D). However, neglecting the concentrations 0.1 and 0.01 led to better efficiency (E= 2.0 and 1.98) for *18S* and *ACTB*, respectively (data not shown).

qPCR efficiency in cells transfected with si-E2F6

For further optimization of RT-qPCR, 10-fold serial dilutions of cDNA from MCF10A were used in RT-qPCR. MCF10A cells were already transfected with either scrambled control (non-specific siRNA) or si-E2F6. Absolute Ct values of *18S* and *E2F6* mRNA were calculated. (Figure 2) demonstrates that the Ct value of *18S* was stable in both cells transfected with si-E2F6 (E= 2.02) and negative control cells (E= 2.0). This finding confirms the suitability of using *18S* as a reference gene.

Concerning *E2F6*, the efficiency was low (E= 3.17) in the transfected cells compared to the control cells (E= 2.7) as *E2F6* is well known to be regulated in the cell cycle (23, 24). Deleting the lowest concentration of E2F6 (1:100,000) improved the amplification efficiency to (E=2.2) in control cells and (E= 2.5) in cells transfected with si-E2F6 (Data not shown). Furthermore, deleting both 1: 10,000 and 1: 100,000 from the standard curve resulted in an efficiency of E= 2.1 for the E2F6 in control cells and E= 2.3 in cells transfected with si-E2F6.

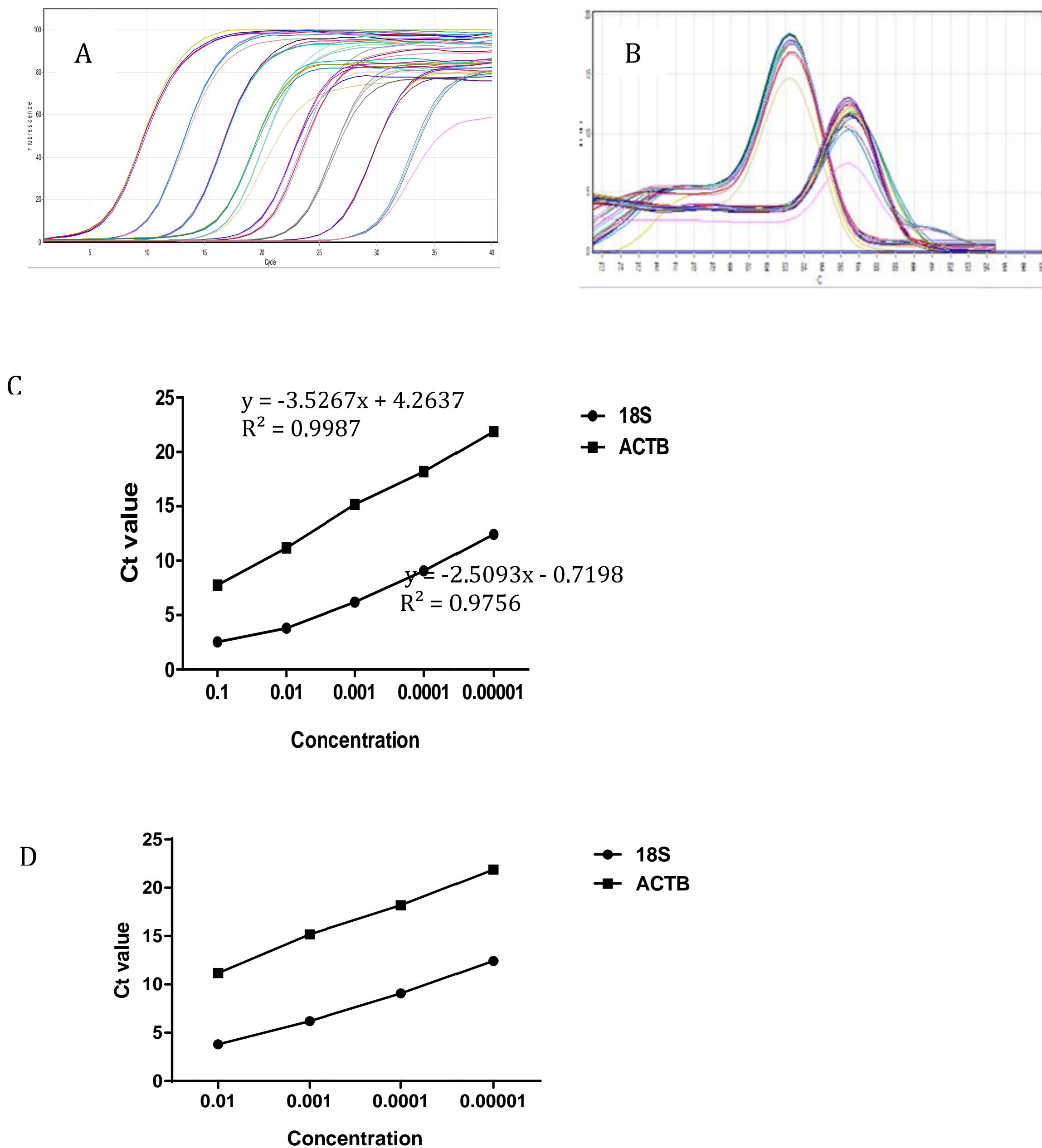

Figure (1): Expression of *ACTB* and *18S* studied in a cancer cell line without treatment. MDA-MB-468 cells were pelleted, and then RNA was extracted and converted to cDNA.10-fold serial dilutions were made from cDNA and subjected to RT-qPCR. The Ct values of *18S* and *ACTB* were investigated into triplicate technical repeats. A- Fluorescence signal of RT-qPCR. The reaction was monitored with SYBR Green I. B- Clean Melt curve for the above genes without any primer dimmers, but only reference genes were amplified. Each peak represents the melt curve in triplicate. The peaks on the left are the melt curves of *18S*, whereas the smaller peaks on the right are the melt curves of *ACTB*. C- Standard curve shows the relationship between Ct value of either *18S* (lower line) or *ACTB* (above line) with 5 dilution series of cDNA and the trend line equation is shown. D- Standard curve plotted with 4 dilution series of cDNA versus Ct values.

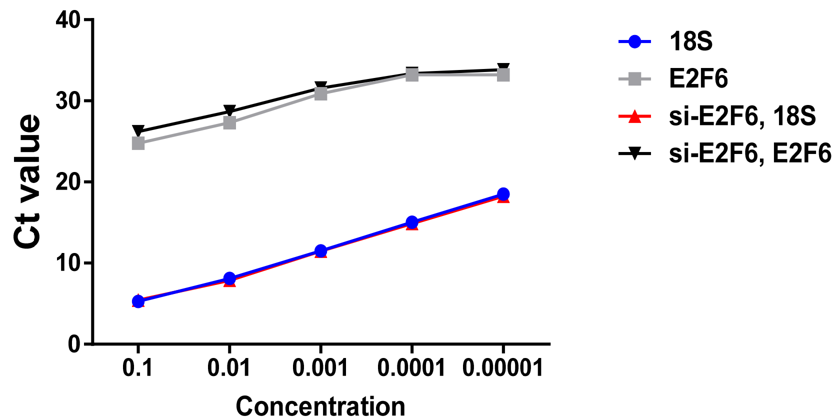

Figure (2): Standard curve shows the amplification efficiency of *18S* compared to *E2F6*.

MCF-10A cells were transfected with si-E2F6 or non-specific siRNA, after 48h cell pellets were collected. 10-fold serial dilutions of extracted cDNA were made and tested by RT-qPCR to measure absolute Ct values of *18S* and *E2F6* mRNA level in transfected and scrambled control cells. Prism software was used to plot the standard curve, which shows the correlation between Ct value and cDNA concentration**.**

Comparing the expression stability of *18S* and *ACTB* in transfected cells

For further analysis of the expression stability of the reference genes under study, MCF-7, MDA-MB-231, T47D, HeLa and HEK293 cell lines were transfected for 48h with either siRNA specific for *E2F6* or scrambled control (siRNA not specific for any gene). Total RNA was extracted from the above cells and converted to cDNA. cDNA was diluted 1/20 and subjected to RT-qPCR to amplify *18S* or *ACTB*. Software analysis of RT-qPCR reveals the absolute Ct values of the aforementioned reference genes. We found that absolute Ct values of *ACTB* were fluctuated and a significant difference was noticed between cells depleted of *E2F6* and negative control cells, except MDA-231 cells where the difference was not statistically important (Figure 3). In contrast, the Ct values of *18S* were approximately similar in cells transfected with si-E2F6 or scrambled control. According to these data, *18S* is much better than *ACTB* to be used for normalization of RT-qPCR especially in gene knockdown experiments.

Calculating relative expression of *E2F6* mRNA level remaining after its knockdown

The relative expression of *E2F6* mRNA remaining after its depletion in the studied cell lines was measured using the method described by Chum *et al.* (25). Either *18S* or *ACTB* was used as a reference gene for normalization of RT-qPCR. Our findings show noticeable differences in *E2F6* relative expression when using 18S or *ACTB* for normalization. While normalizing with *18S* revealed a great decrease in *E2F6* level demonstrating successful gene knockdown, *ACTB*

normalization, on the other hand, showed relatively low reduction in the mRNA level in transfected cells. This was clear in MDA-MB-231 cells transfected with si-E2F6#2 or si-E2F6#3. Concerning MCF-7 cells, normalization with *18S* showed better *E2F6* knockdown with all si-E2F6 than *ACTB* normalization, which showed decrease in *E2F6* mRNA level to the quarter in cells transfected with si-E2F6#1. Regarding T47D cells, normalizing with *18S* gave better gene knockdown compared to *ACTB*. Relative expression of *E2F6* mRNA remaining after gene knockdown was similar when normalize with *ACTB* or *18S* in both HEK293 and HeLa cell lines, indicating that other reference genes might be needed to be tested for their expression stability in those cells for normalization of RT-qPCR.

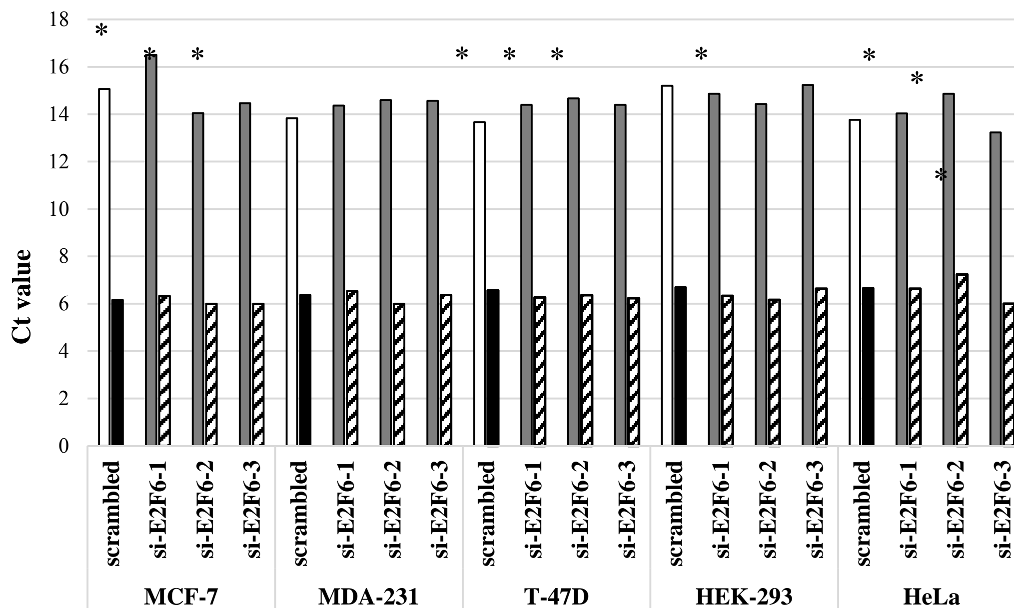

Figure (3): Absolute Ct values of *ACTB* and *18S* studied in cDNAs of some cell lines.
MCF-7, MDA-MB-231, T47D, HeLa and HEK293 cell lines were transfected with specific si-E2F6 for 48h. The cells were pelleted, and then RNA was extracted and converted to cDNA, which was diluted into 1/20 and subjected to RT-qPCR. The expression of *18S* and *ACTB* was investigated into triplicate technical repeats. A Graph shows the Ct values of *18S* and *ACTB*. Gray and streaked bars represent the absolute Ct values of *ACTB* and *18S*, respectively in cells depleted of *E2F6* compared to the white and black bars, which indicate the Ct values of *ACTB* and *18S*, respectively in the negative control cells. The stars represent a significant difference of $p<0.05$ between cells transfected with specific siRNA and non-specific siRNA in Mann-Whitney U test.

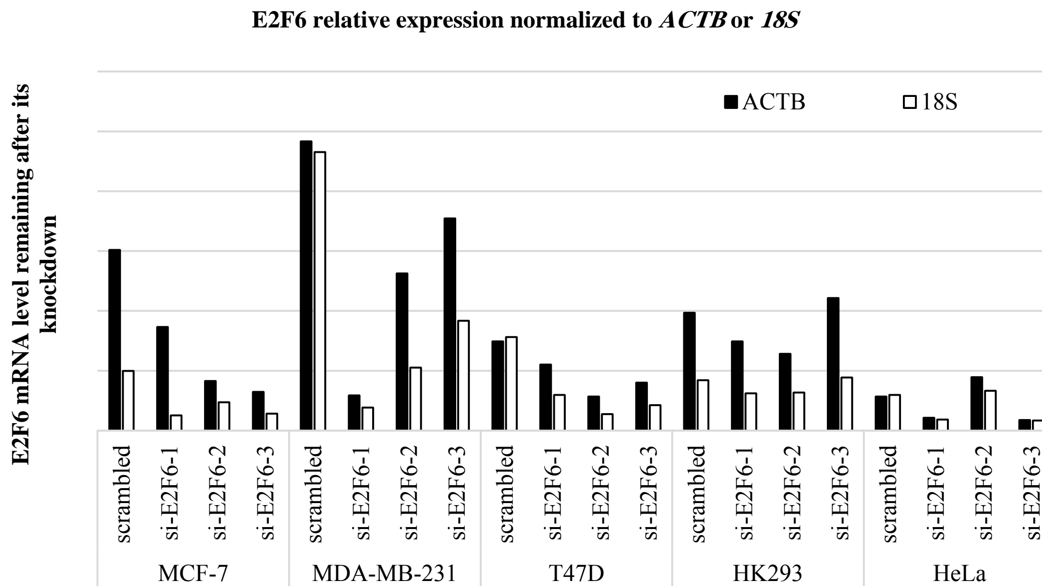

Figure (4): E2F6 mRNA level remaining after its knockdown in five cell lines normalized with *18S* or *ACTB*.
MCF-7, MDA-MB-231, T47D, HeLa and HEK293 cell lines were transfected with specific si-E2F6 for 48h. The cells were pelleted, and then RNA was extracted and converted to cDNA, which was diluted into 1/20 and subjected to RT-qPCR. Relative expression of *E2F6* remaining after its knockdown in the above cell lines was normalized with the reference genes either*18S* or *ACTB*. Black bars represent *E2F6* mRNA remaining level normalized with *ACTB*, whereas the white bars indicate the remaining *E2F6* mRNA level normalized with *18S*.

Discussion

Using a more stably expressed reference gene or genes that are expressed independently of factors influencing the tissue is critical for each experimental set up. It is well known that the expression of ideal reference genes can be different in different cell lines. An important aspect when using reference genes is the primer design. In this study, either the forward or reverse primer of each gene was placed at the junction between two exons to avoid contamination with amplified gDNA. Presence of intron in an amplicon increases its length and leads to incomplete transcript that is devoid of reverse primer sequence, thereby inhibiting further amplification (7). It is known that *ACTB* has pseudogenes as investigated by BLAST search; we were able to avoid this problem (preventing gDNA amplification) by placing the primers to target 5'UTR (untranslated region). To reduce the possibility of using reference genes that are co-regulated, reference genes situated on different chromosomes and involved in various basic cellular processes should be included (7). The target gene in this study, *E2F6*, is located on chromosome number 16, whereas *ACTB* and *18S* are located on chromosomes 7 and 22, respectively (NCBI; bioinformatics). Our findings demonstrate that *ACTB* is a suitable reference gene at least in MDA-MB-468 cell line without treatment. However, *18S* amplification efficiency improved when removing 0.1 and 0.01 concentrations from the standard curve (Figure 1). Saturation of qPCR

reactions at higher cDNA quantities or non-detectable amplicons at the lowest cDNA amounts affected the efficiency of *18S*. Our findings agree with that of (12) in that *18S* was more stably expressed in different cell lines without transfection, but we disagree with them as *ACTB* expression was fluctuated in those untransfected cells (Figure 3, gray and streaked bars). A study was performed by Liu *et al.,* (12) to identify the most stably expressed reference genes in normal breast and breast cancer cell lines using the four frequently used algorithms: geNorm, NormFinder, Best Keeper and comparative delta Ct. Varying results were shown with these methods because each technique has its own strategy to assess gene stability. In that study, NormFinder and comparative delta Ct found that *18S rRNA* and *ACTB* were the most stable expressed reference genes, whereas geNorm and Best Keeper suggested that *18S rRNA* and *PUM1* were the best reference genes. Using Reffinder, *18S rRNA, ACTB* and *PUM1* were identified as the most stably expressed reference genes (12). Therefore, to avoid this discrepancy in the reference gene stability in different cell lines, selecting the most stably expressed reference gene has become a pre-request for each experiment set-up. This study is the first to investigate the influence of target gene knockdown exemplified by *E2F6* on the stability of expression of *18S* and *ACTB* in various cancer cell lines. To assess the expression stability of reference genes, we transfected different cancer cell lines either with siRNA specific for *E2F6* or non-specific siRNA. Three different si-E2F6 was used, with each targeted different areas of the gene sequence to get consistent RNAi response and to augment confidence in experimental findings (26). However, Liu *et al.* (12) investigated the influence of transfection reagents using either Lipofectamine 2000 reagent or X-tremeGENE HP DNA transfection reagent without using siRNA. In this study, the cell lines transfected with either specific si-E2F6 or siRNA not specific for any gene and normalized with *ACTB* showed a significant difference in absolute Ct values measured by RT-qPCR. However, no significant difference in Ct values between si-E2F6 transfected cells and scrambled control was reported upon using *18S* for normalization of RT-qPCR in most tested cell lines (Figure 3). *E2F6* gene was selected as a target of siRNA mediated gene knockdown in this study because E2F6 protein has been suggested to exert its physiological role throughout cell cycle progression (27). E2F6 was found to control genes that are involved in the pathogenesis of cancer and regulating heredity and chromatin structure (28). Furthermore, E2F6 has been revealed to repress a number of target genes in human cancer cell lines by competition with activating E2Fs (28). *E2F6* belongs to the E2F family of transcription factors, which is comprised of eight genes that encode nine major proteins: E2F1, 2, 3 (a, b), 4-8. The family members can be classified as transcriptional activators or repressors depending on their structure and functions (29). To calculate the relative expression of the target gene *E2F6*, either *ACTB* or *18S* were used for normalizing RT-qPCR data. The findings show noticeable differences in *E2F6* relative expression when using *18S* or *ACTB* for normalization. While normalizing with *18S* revealed great decrease in *E2F6*

transcript level demonstrating successful gene knockdown, *ACTB* normalization, on the other hand, showed relatively low reduction in the mRNA level in transfected cells. An increase in aggressiveness of breast cancer cell lines has been determined to affect which optimal reference gene to be used for RT-qPCR normalization (30). This study concludes that the reference gene has to be chosen cautiously for each experimental set up and for each cell line in order to obtain accurate findings.

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