RESEARCH ARTICLE

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Validation of internal control genes for quantitative real-time PCR under different experiment conditions in Multiple Myeloma

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ABSTRACT
Multiple myeloma (MM) is a cancer of malignant plasma cells in the bone marrow which might lead to the development of one or more clinical manifestations such as bone destruction, anemia, renal insufficiency etc. Gene expression analysis using quantitative real-time PCR (qRT-PCR) is imperative to understand the developmental mechanisms of MM. Housekeeping genes (HKGs) are commonly used as endogenous controls to normalize quantitative real-time PCR (qRT-PCR) data for gene expression analysis. However, recent studies argue that the expression of HKG genes may vary under certain experimental condition. In addition, no studies have been found on the expression analysis of HKGs by qRT-PCR in MM. Therefore, the present study was designed to validate reference genes for qRT-PCR normalization through observing the effects of hypoxia, serum stimulation and Myc inhibition on the expression of five HKGs (18S, ACTB, B2M, GAPDH, and TBP) in three different myeloma cell lines (ANBL-6, IH-1 and INA-6). Four different approaches (Best Keeper, ΔCt approach, GeNorm, and NormFinder) followed by comprehensive methods were used for the evaluation and selection of reference gene. Most stable expression of 18S in hypoxic and serum stimulation experiment while constant expression of B2M in Myc inhibition made them an excellent combination to normalize qRT-PCR data in gene expression analysis in MM.

Key words: Multiple myeloma, reference genes, real-time PCR, hypoxia, serum stimulation, Myc inhibition

Introduction

Multiple myeloma (MM) is hematologic cancer, characterized by excess malignant plasma cells in the bone marrow (BM). It is the second most frequent malignancy of blood after non-Hodgkin lymphoma in the United States and eventually resulted from ~2% of cancer deaths worldwide in 2012 (Kumar, 2010; Holien et al., 2015). The malignant plasma cells secrete a monoclonal immunoglobulin (M-protein) leading to various clinical manifestations such as bone destruction through lytic bone lesions; hypercalcemia by excessive bone resorption and suppression of new bone formation; anemia due to suppression of normal erythropoiesis; renal insufficiency due to the toxic effects of the monoclonal immunoglobulin secreted by myeloma cells (Mitsiades et al., 2007; Kumar, 2010). MM is still an incurable disease with a survival time of 3-4 years after high-dose chemotherapy and autologous stem cell transplantation (Kyle & Rajkumar, 2009). Therefore, it is essential to understand the molecular mechanism of MM for therapeutic targets and clinical outcome improvement. Thus, many researchers are trying to identify the key genes and their expression pattern to understand biological mechanisms such as invasion, metastasis, angiogenesis, and signal transduction leading to the development of MM (Magrangeas et al., 2003; Zhan et al., 2006; Broyl et al., 2010) corroborating the importance of gene expression analysis in MM research including prognosis, classification, potential therapeutic target and drug development.

Assessment of the molecular state of various cells and tissues through gene expression analysis is widely used to know of complex regulatory networks involved in disease initiation. There are several methods including microarray, transcriptome (RNA-seq) and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) are commonly used to quantify gene expression (Caradec et al., 2010; Rajkumar et al., 2015). Both microarray and RNA-seq allow comparative expression analysis of thousands of genes in specific sets of RNA. However, RNA-seq based on next-generation sequencing is replacing microarrays for the quantification of transcripts abundance as it has the ability to detect novel transcripts, alternative splice sites, allele-specific expression and sequence variation (Costa et al., 2013). On the

other hand, qRT-PCR is a rapid, sensitive, reproducible, accurate and reliable method for quantification of mRNA expression of the genes of interest in various experimental conditions and clinical applications. Furthermore, real-time PCR is widely used to reduce cost, validate the transcriptome or microarray data and in clinical diagnoses as it relies on a set of target genes (Chen et al., 2015; Lemma et al., 2016). Normalization of samples is exigent in gene expression analysis to minimize the errors resulting from limitations associated with qRT-PCR such as inherent variability of RNA, RNA quality, and reverse transcription and PCR efficiencies (Huggett et al., 2005). Housekeeping genes encode proteins that provide basic and essential cellular functions for the cells are recognized as reference genes and commonly used for normalization of gene expression analysis in qRT-PCR (Vandesompele et al., 2002). Ideally, the HKGs used as endogenous control should be abundantly expressed and have stable expression across tissues, cell types, and various experimental conditions. However, the expression of these housekeeping genes displays significantly different expression level under different experiential conditions or in various tissues. Therefore, validation of the reference gene is crucial for the accurate measurement of gene expression in qRT-PCR as inappropriate normalization can result in inadequate quantification and erroneous gene expression profile (Liu et al., 2015). As a result, recently, a number of studies have been conducted to evaluate the expression stability of candidate reference genes under certain experimental conditions, or in different tissues or cell lines for normalization of qRT-PCR analysis (Silver et al., 2006; Caradec et al., 2010; Chen et al., 2015; Liu et al., 2015; Lemma et al., 2016; Pereira-Fantini et al., 2016).

For mammalian cell culture, serum is frequently added to the medium and most human myeloma cell lines (HMLC) grow in medium supplemented with fetal calf serum (FCS) or human serum (HS). Serum contains different biomolecules that have the ability to promote different biological activities of cells through changes in expression of many genes including transcription factors, extracellular matrix proteins, enzymes and others (Gstraunthaler, 2003). Thus, the expression of housekeeping genes might be affected by adding serum into the culture. Cells are also affected by low oxygen concentration or hypoxia. In response to hypoxic conditions, cancer cells can survive and proliferate by changing the transcription of genes that can increase angiogenesis while hypoxia generally leads to cell cycle arrest in normal cells resulting in apoptosis (Johnson et al., 2008). So, many genes including HKGs can have differential expression pattern in response to hypoxic conditions compared to normal condition. Myc protein is a transcription factor that activates several biological events including regulation of cell proliferation, apoptosis, and differentiation, and its aberrant expression is often seen in several human cancers. Inhibition of Myc can induce cell cycle arrest and apoptosis in myeloma cells (Holien et al., 2015). So, it may also have the ability to change the expression of numerous numbers of genes in MM.

To date, there are no studies available about validation of endogenous gene under different experimental conditions for HMCLs. Here, for the first time the expression of five most common housekeeping genes such as 18S ribosomal RNA (18S), actin beta (ACTB), beta-2-microglobulin (B2M), glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and TATA-box binding protein (TBP) were analyzed to investigate their robustness for qRT-PCR normalization in different experimental conditions such as hypoxia, serum induction (SI) and Myc inhibition in three different myeloma cell lines.

Materials and Methods

Cells and culture conditions

Human myeloma cell lines ANBL-6 and INA-6 cells were the kind gifts from Dr. Diane Jelinek (Mayo Clinic, Rochester, MN, USA) and Dr. Martin Gramatzki (University of Erlangen – Nuremberg, Erlangen, Germany), respectively while IH-1 cells were established in-house from MM patients (Hjertner et al., 2001). Cells were cultured in complete medium consisting RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 100 µg/mL L-glutamine and 20 µg/mL gentamicin. ANBL-6 and INA-6 were grown in RPMI containing 10% heat-inactivated fetal calf serum (FCS) (HyClone, Logan, UT, USA) while IH-1 cells which were maintained with 10% heat-inactivated human serum (HS) (Blood Bank St. Olav's University Hospital, Trondheim, Norway). Cell lines were maintained in media containing 1ng/mL IL-6 (R&D Systems, Abingdon, UK) at 37°C in a humidified environment (21% O_2 and 5% CO_2/air) and replenishment was performed twice a week.

Experiment conditions

Hypoxic conditions

Countess Cell Counter (Invitrogen) was used for counting of myeloma cell after washing with phosphate-buffered saline (PBS) followed by 5×10^5 live cells were seeded in six-well plates containing RPMI 1640 medium supplemented with 10% FCS (ANBL-6 and INA-6) or HS (IH-1). After that cells were incubated under hypoxic conditions (1% O_2 and 5% CO_2/air) for different time (24, 48, 72 hours) intervals along with seeding same numbers of cells under normoxic conditions (21% O_2 and 5% CO_2/air) for 24 hours as control (Muz et al., 2014) followed by RNA extraction from the cells.

Serum induction experiment

Myeloma cells were completely separated from media and washed three times in PBS prior to seeding in RPMI 1640 medium containing 0.5% FCS or HS supplemented with 100 µg/mL L-glutamine, 20 µg/mL gentamicin and 1 ng/mL IL-6 followed by incubation at 37°C in a humidified environment of 5% CO_2/air for 24 hours known as starvation period (Schmittgen & Zakrajsek, 2000). Following the starvation period, FCS or HS was added to make media containing 10% FCS or HS and then the cells were cultured in a humidified incubator of 5% CO_2/air at different time point (0, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 hours) for RNA extraction.

Myc inhibition experiment

Myeloma cells washed with PBS for three times after removing media completely followed by cultured in RPMI 1640 medium supplemented with 10 % FCS (ANBL-6 and INA-6) or HS (IH-1) in a humidified environment of 5% CO_2/air. Then 20 µM Myc inhibitor 10058-F4 was added to the culture media after 1 hour incubation period (Holien et al., 2012). After that, the cells were incubated at 37°C in a humidified environment of 5% CO_2/air for the different time periods (0, 1, 3, 6, 12 and 24 hours) to extract RNA.

To extract total RNA from the cells of all experiments the media was completely removed and washed with PBS followed by suspended in 1 ml RPMI supplemented with 2% serum and spin down at 1500 rpm 4°C for 5 min. Then cells were lysed in 350 m1 of buffer RLT supplement with β_2-marceptoethanol (Qiagen, Valencia, CA, USA) and immediately frozen in liquid nitrogen followed by storage at -80°C. Moreover, equal numbers of cells were used in all the experiment for RNA isolation through counting with Countess Cell Counter. Experimental conditions were performed in triplicates and the experiment was repeated twice.

Total RNA extraction and quality control

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions and extracted RNA was treated with Dnase I (Sigma-Aldrich, 3050 Spruce St, St. Louis, MO 63103) to avoid amplification of genomic DNA. The concentration and purity of RNA were estimated using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc., USA) while the integrity of total RNA was assessed by 1.2% agarose gel electrophoresis.

cDNA synthesis

High Capacity RNA-to-cDNA Kit (Applied BioSystems, Foster City, California, United States) was used according to manufacturer's protocol to generate complementary DNA (cDNA) from RNA template through reverse transcription. About 500 ng of RNA was reverse transcribed using 1 µl 20x RT enzyme mix, 10.0 µl 2x RT buffer and filled up by RNase-free water to a total volume of 20 µl reaction mixture, and incubated for 10 min at 25°C, 120 min at 37°C followed by 85°C for 10 min. Then the residual RNA was degraded by another incubation step at 37°C for 20 min after adding 1 µl RnaseH and stored at -20°C. Elimination of residual genomic DNA was confirmed by PCR amplification of RNA samples in the absence of cDNA.

Primer design

Five common housekeeping genes including 18S, ACTB, B2M, GAPDH, and TBP were selected to validate their expression pattern during various experimental environments. These housekeeping genes are constitutively expressed and show independent function in cellular maintenance. Specific primers for four selected HKGs (18S, ACTB, B2M, and GAPDH) and hepatocyte growth factor (HGF) as a gene of interest were taken from different studies (Xin et al., 1999; Schmittgen & Zakrajsek, 2000; Malarstig et al., 2003; Joosen et al., 2006; Ramani et al., 2011). The primers for other housekeeping gene TBP were received from our laboratory (Table 1). The primers for the genes were purchased from Sigma, Life science (3050 Spruce St, St. Louis, MO 63103). Specificity of all the primers was checked through PCR

Table 1. *Sequences and different parameters of the primers used in qRT-PCR analysis.*

Gene Name	Gene Description	GeneBank Accession Number	Primer Pairs	Efficiency (%)	PCR length (bp)
18S	18S ribosomal RNA	NR_003286	F: 5'-GTAACCCGTTGAACCCCATT-3' R: 5'-CCATCCAATCGGTAGTAGCG-3'	101.9	151
ACTB	Actin beta	NM_001101	F: 5'- AGAAAATCTGGCACCACACC -3' R: 5'- AGAGGCGTACAGGGATAGCA -3'	94.8	188
B2M	Beta-2-Microglobulin	NM_004048	F: 5'-AGCGTACTCCAAAGATTCAGGTT -3' R: 5'-TACATGTCTCGATCCCACTTAACTA-3'	100.1	295
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	F: 5'-GAAGGTGAAGGTCGGAGTC -3' R: 5'-GAAGATGGTGATGGGATTTC-3'	93.9	226
TBP	TATA-box-binding protein	NM_003194	F: 5'-TTGCTGCGGTAATCATGAGG-3' R: 5'-GCCAGTCTGGACTGTTCTTC-3'	96.4	109
HGF	Hepatocyte growth factor	NM_000601	F: 5'- CAATAGCATGTCAAGTGGAG -3' R: 5'- CTGTGTTCGTGTGGTATCAT -3'	104.6	180

followed by electrophoresis on a 1.5% agarose gel and melting curves analysis during real-time PCR. To determine the efficiencies of the primer five serially diluted cDNA in a qRT-PCR assay was used to obtain the standard curve on Microsoft Excel. The correlation coefficient (R^2) and slope values were obtained from the standard curve using the following formula: $E = [10^{-(1/slope)} -1] \times 100\%$.

Real-time quantitative PCR

Quantitative Real-time PCR using SYBR green technology was performed by a StepOnePlus real-time PCR System from Applied Biosystems (AB International, CA). The qRT-PCR reaction mix was prepared by adding 12.5 µl of SYBR green PCR master-mix (Applied BioSystems, CA), 1.0 µl of 5 µM forward primer, 1.0 µl of 5 µM reverse primer, 10 ng cDNA and RNase free water to make a total volume of 25 µl. The PCR program was set up with an initial heat activation step at 95°C for 10 min. Then, 40 cycles of thermocycling were performed with a denaturation step at 95°C for 15 sec, an annealing step at 60°C for 30 sec and an elongation step at 72°C for 30 sec. Fluorescence was measured at the end of each step and melting curves were used to determine the specificity of PCR products.

Statistical analysis of reference gene selection

To select a suitable internal control gene for qRT-PCR normalization, three different visual basic application of Microsoft Excel such as GeNorm, NormFinder, BestKeeper, and standard comparative Ct method (ΔCt) were used in this study (Vandesompele et al., 2002; Andersen et al., 2004; Pfaffl et al., 2004; Silver et al., 2006). For both GeNorm and NormFinder analysis, raw Ct values from qRT-PCR were transformed into relative quantities using the following formula: $2^{-\Delta Ct}$, where highest Ct was used as calibrator. In contrast, raw Ct values were directly used for BestKeeper, ΔCt and PCR efficiency analysis. GeNorm calculates average gene expression stability value (M), where lower M value means more stable reference gene. The optimal number of internal genes required for accurate normalization is also determined by calculating the pairwise variation (V_n/V_{n+1}) using GeNorm. On the other hand, NormFinder evaluates suitable control gene on the basis of their expression stability in a sample set. The selection of control genes by BestKeeper based on the coefficient of variation and standard deviation whereas ΔCt determines the most stable gene by comparative relative expression. Moreover, a comprehensive ranking was calculated by the geometric mean of individual ranking obtained from four algorithms for three cell lines under various experimental treatments (Xiao et al., 2014; Pereira-Fantini et al., 2016). Finally, another consensus ranking was established for each experimental condition by calculating the geometric mean based on the ranking value of each reference gene considering all algorithms and cells. In order to validate the selected HKG relative expression level of HGF was calculated by $2^{-\Delta\Delta Ct}$ method under hypoxia and serum stimulation experimental conditions using ANBL-6 cell lines (Livak & Schmittgen, 2001).

Results

RNA quality

Good quality RNA is necessary for gene expression analysis, therefore total RNA extracted from different cells under various experimental conditions were examined spectrophotometrically for measuring purity and integrity. All RNA samples had an A260/A280 absorbance ratio greater than 1.8, indicating protein free RNA, and at the absorbance ratios at 260/230 nm was over 1.9, indicates phenol and ethanol free pure RNA (data not shown). In addition, well-defined rRNA bands in 1.2% agarose gel electrophoresis confirmed that the total RNA retained its integrity of the RNA (Figure 1). Moreover, no band was found in agarose gel electrophoresis when PCR was performed using crude RNA suggests genomic DNA contaminant free RNA (Figure 2).

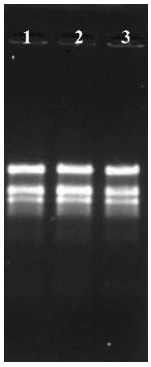

Figure 1. *Agarose gel electrophoresis analysis of total RNA extracted from different myeloma cells such as (1) ANBL-6, (2) IH-1 and (3) INA-6.*

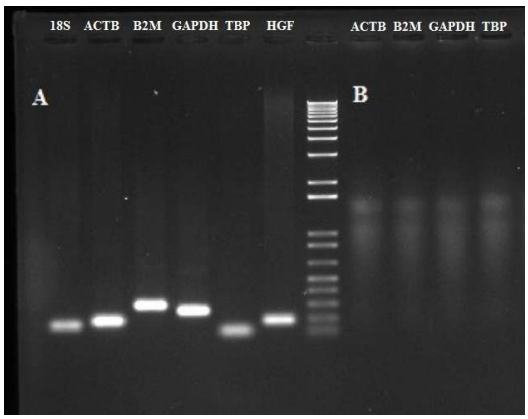

Figure 2. *qRT-PCR amplification specificity and checking DNA contamination in RNA. (A) cDNA was used to amplify PCR products designed for 18S, ACTB, B2M, GAPDH, TBP and HGF, (B) RNA was used to amplify PCR products designed for ACTB, B2M, GAPDH, and TBP.*

PCR specificity and efficiency

The amplification specificity of five HKGs and HGF genes were confirmed by the presence of a single band with expected size in agarose gel electrophoresis (Figure 2A). Moreover, single peaks obtained from dissociation curve analysis ensured specific product amplification by the primers (Figure 3). The efficiency of PCR reaction for each primer pair was from 93.9 to 104.6% and the correlation co-efficiency (R^2) ranged from 0.99 to 0.996 (Table 1; Figure 4).

Box-whisper analysis

In order to determine the stability of five HKGs for the myeloma cells under different experimental conditions at different time points, an equal amount of cDNA was used for amplification by real-time PCR. The expression levels of the candidate genes were quantified by their mean Ct values which were ranged from 9.40 to 26.99, where the majority were lying between 17.0 to 19.37 (Figure 5). The expression level of 18S was relatively high with low Ct values while TBP showed the lowest expression level with high Ct values across all the cells under various experimental conditions. The rest of the genes (ACTB, B2M, and GAPDH) were moderately expressed. On the contrary, TBP showed the least variation in gene expression with the coefficient of variation (CV) of ~2.2% for all cell lines. In contrast, 18S showed maximum variation in their expression levels with CV value of >5% both in ANBL and IH-1 cell lines while in INA-6 cells, GAPDH was exhibited highest variance with CV value of 6.4% across all experimental conditions. Above results clearly indicate the expression variation of candidate genes in different cells under various environmental conditions. Thus, inconsistent expression of the candidate genes suggests the importance of identifying an appropriate internal control gene for normalization of gene expression analysis using qRT-PCR in human myeloma cell lines.

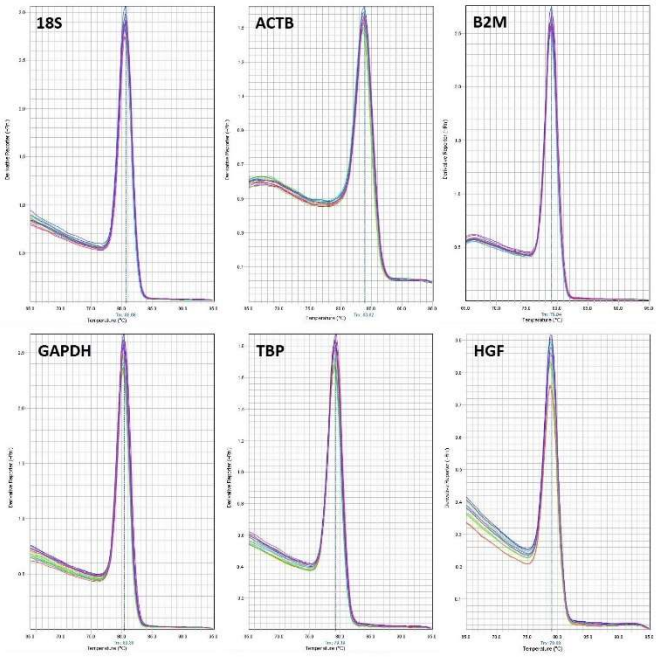

Figure 3. *Specificity of qRT-PCR amplification. Dissociation curves of the six genes (18S, ACTB, B2M, GAPDH, TBP and HGF) with single peak after qRT-PCR reactions.*

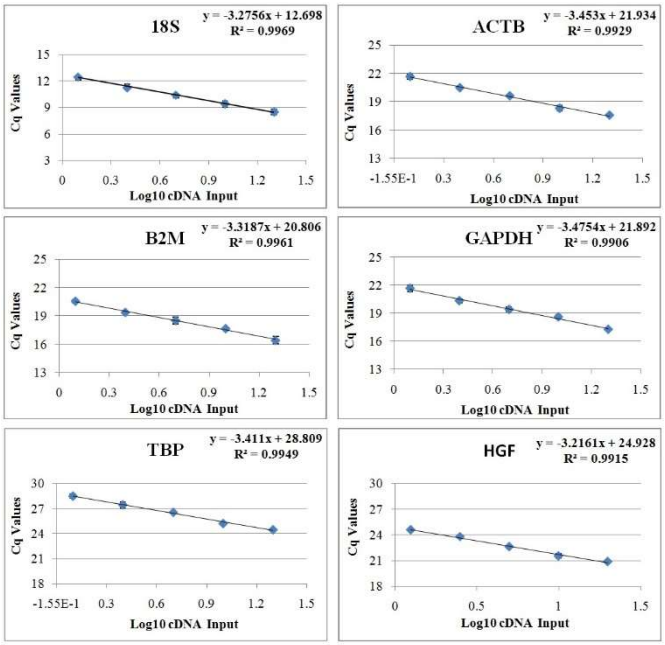

Figure 4. *PCR efficiency based on Standard curve for 18S, ACTB, B2M, GAPDH, TBP and HGF in real-time PCR. The x-axis represents the copies of the cDNA and y-axis represents of the cycle threshold.*

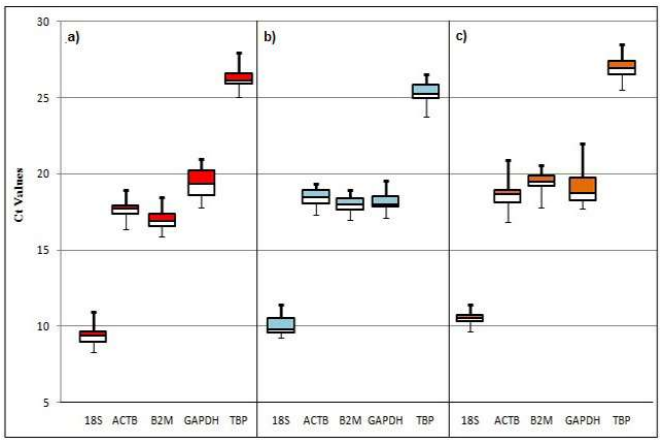

Figure 5. *Expression levels of the candidate reference genes for different experimental conditions in ANBL-6 (a), IH-1 (b) and INA-6 (c) myeloma cells. The expression levels are displayed as cycle threshold (Ct) values for reference genes of the human myeloma cells under different experiment conditions. The central horizontal line across the box denotes the median while the box indicates the 25th and 75th percentile. Whisker caps indicate maximum and minimum values.*

Hypoxia experiment

Four different algorithms (GeNorm, NormFinder, BestKeeper, and ΔCT) were used to evaluate the expression stability of five candidate genes under hypoxic conditions. According to Genorm, ACTB and B2M having lowest average gene expression stability (M value) in ANBL-6 cells while 18S and GAPDH were most suitable reference genes for qRT-PCR normalization both in IH-1 and INA-6 myeloma cell lines (Figure 6a). In addition, NormFinder and ΔCT ranked 18S, GAPDH, and TBP in ANBL-6, IH-1 and INA-6 cells, respectively. B2M was most stably expressed in ANBL-6 while 18S identified as a suitable reference gene in the other two cells by BestKeeper analysis (Table 2).

Serum Induction Experiment

For ANBL-6 cells, B2M appeared as the top-ranked reference gene in all four algorithms under serum induction experiment (Table 3; Figure 6b). 18S was also found to be the most suitable gene according to GeNorm analysis as it gave the most stable pair of genes (Figure 6b). Likewise, 18S was identified as the most stable gene considering in three methods except ΔCT in IH-1 cell lines. ACTB had stable expression considering both GeNorm and ΔCT analysis in IH-1 (Table 3; Figure 6b). In contrasts, another myeloma cell lines INA-6 showed different ranking obtained from the algorithms. B2M was in the top position according to both NormFinder and ΔCT analysis, while according to BestKeeper 18S had the remarkably stable expression with the lowest CV values in INA-6 cells (Table 3). Lower M value of 18S and ACTB indicates their stable expression according to GeNorm analysis (Figure 6b).

Myc Induction Experiment

In Myc induction experiment, B2M turned out to be the best internal genes in ANBL-6 and IH-1 cells while the expressions of 18S showed in INA-6 cells ranked it to be the most stable gene according to BestKeeper, NormFinder and ΔCT approach (Table 4). According to GeNorm, both 18S and ACTB had the lowest M value suggesting their stable expression in ANBL-6. On the other hand, ACTB and B2M were constantly expressed in IH-1 cells whereas B2M and TBP in top place in INA-6 cell lines when considering GeNorm (Figure 6c).

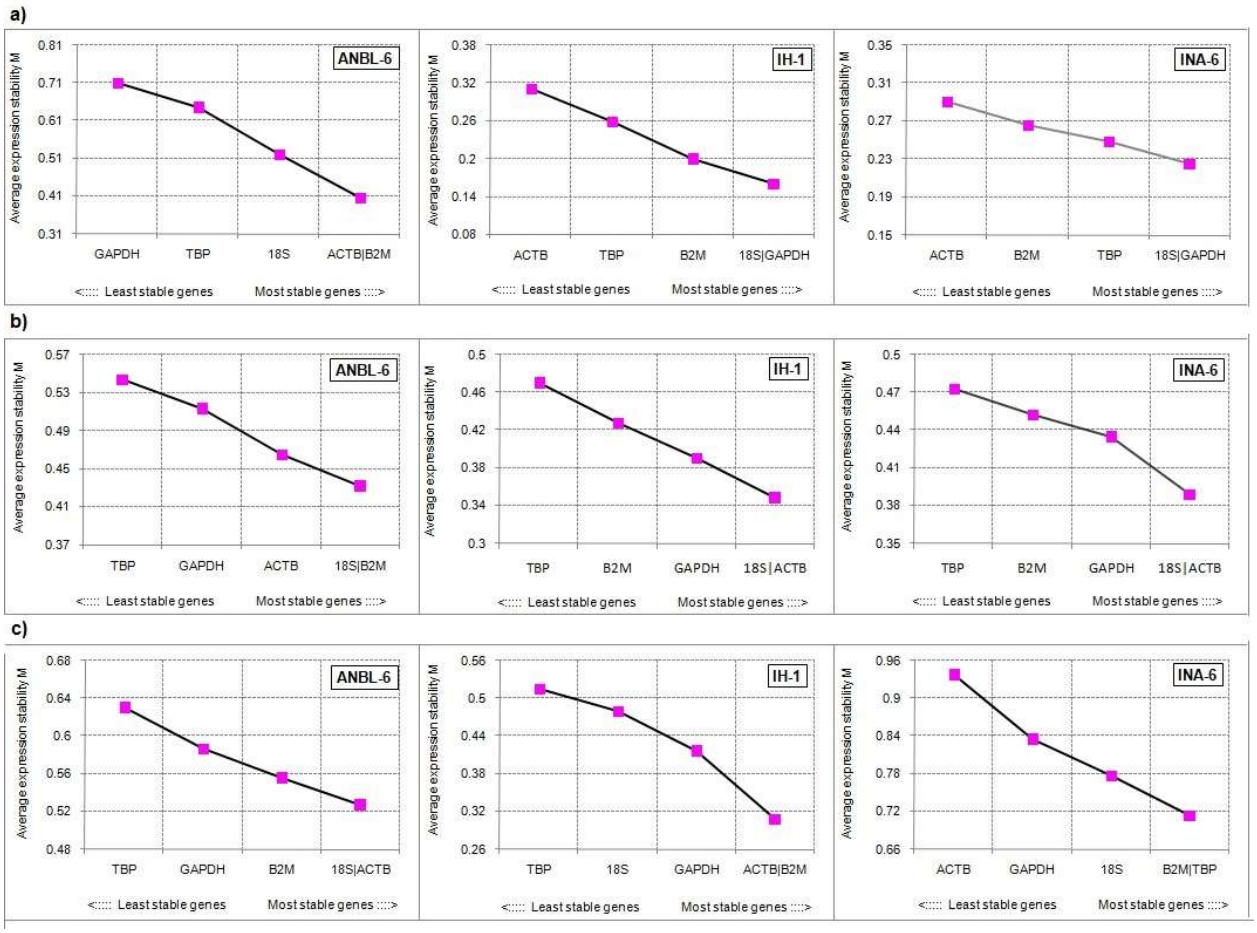

Figure 6. *Average expression stability values (M) of five candidate genes in myeloma cells under hypoxia (a), SI (b) and Myc induction (c) experiment conditions. Expression stability (M) value of the reference genes for human myeloma cell lines were calculated by GeNorm algorithm for hypoxic conditions, serum stimulation experiment, Myc inhibition experiment in ANBL-6, IH-1 and INA-6 cells. The lowest expression stability value indicates more stable gene.*

Table 2. *Expression stability of candidate reference genes for hypoxia in myeloma cells as calculated by NormFinder, BestKeeper and ΔCt.*

	ANBL-6			IH-1			INA-6		
HKG	**NF (SV)**	**BK (SD)**	**ΔCT (SD)**	**NF (SV)**	**BK (SD)**	**ΔCT (SD)**	**NF (SV)**	**BK (SD)**	**ΔCT (SD)**
18S	1(0.234)	2(0.21)	1(0.65)	4(0.169)	1(0.13)	3(0.31)	3(0.140)	1(0.08)	3(0.29)
ACTB	2(0.332)	3(0.34)	2(0.68)	5(0.240)	5(0.47)	5(0.39)	5(0.196)	5(0.34)	5(0.33)
B2M	4(0.365)	1(0.17)	3(0.70)	2(0.112)	3(0.23)	2(0.28)	2(0.120)	3(0.23)	2(0.28)
GAPDH	5(0.477)	5(0.60)	5(0.72)	1(0.078)	2(0.15)	1(0.26)	4(0.191)	2(0.21)	4(0.33)
TBP	3(0.346)	4(0.58)	4(0.81)	3(0.151)	4(0.28)	4(0.32)	1(0.015)	4(0.24)	1(0.24)

Table 3. *Expression stability of candidate reference genes for serum induction experiment in myeloma cells as calculated by NormFinder, BestKeeper and ΔCt.*

	ANBL-6			IH-1			INA-6		
HKG	**NF (SV)**	**BK (SD)**	**ΔCT (SD)**	**NF (SV)**	**BK (SD)**	**ΔCT (SD)**	**NF (SV)**	**BK (SD)**	**ΔCT (SD)**
18S	3(0.292)	3(0.40)	3(0.56)	1(0.171)	1(0.16)	2(0.430)	2(0.219)	1(0.22)	2(0.46)
ACTB	2(0.222)	4(0.54)	2(0.51)	2(0.173)	3(0.29)	1(0.429)	3(0.223)	2(0.23)	3(0.47)
B2M	1(0.151)	1(0.29)	1(0.47)	4(0.279)	5(0.38)	4(0.507)	1(0.210)	3(0.26)	1(0.45)
GAPDH	4(0.315)	5(0.61)	4(0.58)	3(0.204)	2(0.26)	3(0.449)	4(0.238)	4(0.32)	4(0.48)
TBP	5(0.327)	2(0.39)	5(0.59)	5(0.307)	4(0.34)	5(0.533)	5(0.272)	5(0.36)	5(0.50)

Table 4. *Expression stability of candidate reference genes for Myc inhibition experiment in myeloma cells as calculated by NormFinder, BestKeeper and ΔCt.*

	ANBL-6			IH-1			INA-6		
HKG	**NF (SV)**	**BK (SD)**	**ΔCT (SD)**	**NF (SV)**	**BK (SD)**	**ΔCT (SD)**	**NF (SV)**	**BK (SD)**	**ΔCT (SD)**
18S	3(0.305)	3(0.33)	3(0.63)	4(0.296)	3(0.37)	4(0.55)	1(0.397)	1(0.25)	1(0.884)
ACTB	2(0.280)	2(0.29)	2(0.61)	3(0.227)	2(0.20)	2(0.49)	5(0.635)	5(0.99)	5(1.091)
B2M	1(0.180)	1(0.22)	1(0.55)	1(0.205)	1(0.12)	1(0.48)	2(0.401)	4(0.77)	3(0.894)
GAPDH	4(0.359)	5(0.42)	4(0.67)	2(0.218)	4(0.39)	3(0.49)	4(0.443)	2(0.68)	4(0.927)
TBP	5(0.395)	4(0.40)	5(0.69)	5(0.317)	5(0.46)	5(0.57)	3(0.404)	3(0.76)	2(0.892)

Legend: HKG, Housekeeping gene; NF, NormFinder; SV, Stability value; BK, BestKeeper; SD, Standard deviation.

Determination of the optimal number of candidate genes for normalization by GeNorm

The GeNorm algorithm also gives the pairwise variation ($V = V_n/V_{n+1}$) value to determine the optimum number of reference genes required for accurate normalization. An additional reference gene has no significant contribution to the normalization in qRT-PCR if $V_n/V_{n+1} < 0.15$ (Vandesompele et al., 2002). $V_{2/3} < 0.15$ in hypoxic condition for IH-1 and INA-6 cells indicates that two most stable genes (18S and GAPDH) were sufficient for accurate normalization in qRT-PCR, while four control genes were required for ANBL-6 cells under hypoxic conditions as $V_{4/5}$ was 0.145 (Figure 7a). In serum induction experiment the $V_{2/3}$ values of all myeloma cell lines were below 0.15 according to the pairwise variation calculated by geNorm (Figure 7b). Therefore, these results suggested that two reference genes with the lowest M values would be sufficient for normalization. As shown in Figure 7c, $V_{2/3}$ was lower than 0.15 for IH-1 cells indicates that two genes would be enough for normalization in terms of Myc inhibition experiment. In ANBL-6, three genes were needed as $V_{3/4}$ value was 0.132. In contrasts, more additional genes were required in INA-6 cell

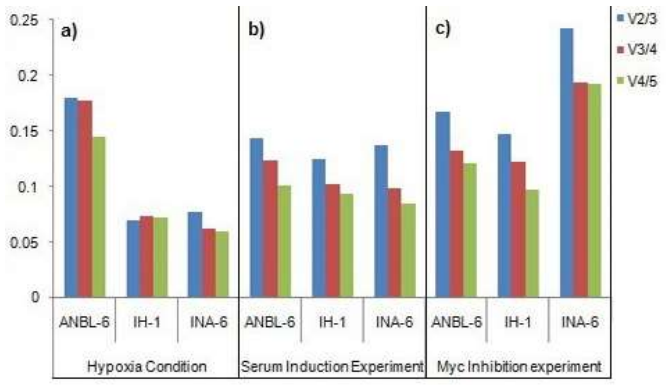

Figure 7. *Pairwise variation (V) of the candidate genes was predicted by GeNorm. The pairwise variation ($V=V_n/V_{n+1}$) was calculated between normalization factor NF_n and NF_{n+1} to determine the optimal number of reference genes required for qRT-PCR normalization in different experimental conditions such as (a) hypoxic conditions, (b) serum stimulation experiment, (c) Myc inhibition experiment for ANBL-6, IH-1 and INA-6 myeloma cell lines. Values under 0.15 indicate that no additional genes are required for the normalization.*

lines for normalization in gene expression analysis under Myc inhibition experiment (Figure 7c).

Comprehensive ranking order

A comprehensive ranking was calculated based on the geometric mean of ranking obtained from the four different algorithms. According to the comprehensive ranking, 18S was the most stable genes both in ANBL-6 and INA-6 cells while GAPDH was the best choice to use as the normalization factor for IH-1 under hypoxic conditions (Figure 8a). For serum induction conditions, B2M was the best housekeeping genes in ANBL-6 for gene expression analysis whereas 18S was showed stable expression both in INA-6 and IH-1 cell lines (Figure 8b). B2M was found as the most stable gene for ANBL-6 and IH-1 in contrast, 18S had a stable expression for INA-6 cells when considering Myc inhibition experiment (Figure 8c). In addition, another consensus ranking was generated by considering all myeloma cells for a specific experimental condition. This ranking was based on the geometric mean of the ranking values from the four approaches of each cell lines under specific conditions. According to the ranking, 18S was the most suitable gene followed by B2M for both hypoxia and SI experiment in qRT-PCR normalization. In contrast, B2M was the best endogenous control followed by 18S under Myc inhibition experiment for myeloma cells in gene expression analysis using qRT-PCR (Figure 9).

Reference gene validation

Expression pattern of HGF was investigated under hypoxia and serum induction experiment in order to validate selected reference genes. HGF, a multifunctional cytokine, stimulates cell growth, cell motility, invasion and metastasis as its expression induced by several factors like hypoxia and serum (Matsumoto & Nakamura, 1996; Ohshima et al., 2002; Kitajima et al., 2008). The expression level of HGF was increased after 24h of hypoxic conditions and afterwards decreased when the most stable gene (18S) and the two top-ranked internal gene (18S and B2M) acted as normalization factor, but the expression pattern of HGF varied when least stable gene, GAPDH, was used for normalization under hypoxia for ANBL-6 cells. The transcripts level of HGF was highest at 24h and then decreased under hypoxic conditions for the best and the two most suitable genes. By contrast, higher expression was observed for HGF after 24h, then decreased at 48h and afterward increased to four-fold at 72h when the least stable gene was employed in gene expression analysis by qRT-PCR under the hypoxic environment (Figure 10a). In serum induction experiment, the transcript level of HGF increased rapidly at 0.5h near to three-fold, then slowly decreased from 1h to 4h and thereafter increased from 6h when using the best and two most suitable genes for ANBL-6 myeloma cells.

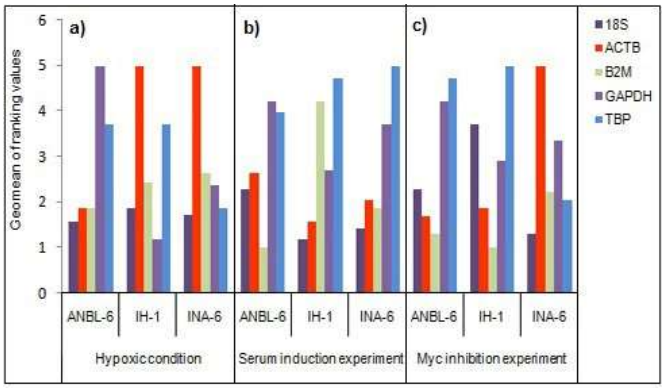

Figure 8. *Comprehensive stability ranking under various experimental conditions for different myeloma cells. The Geometric mean of ranking values obtained from four algorithms was used to compute comprehensive ranking under (a) hypoxic conditions, (b) serum stimulation experiment, (c) Myc inhibition experiment for ANBL-6, IH-1 and INA-6 myeloma cells*

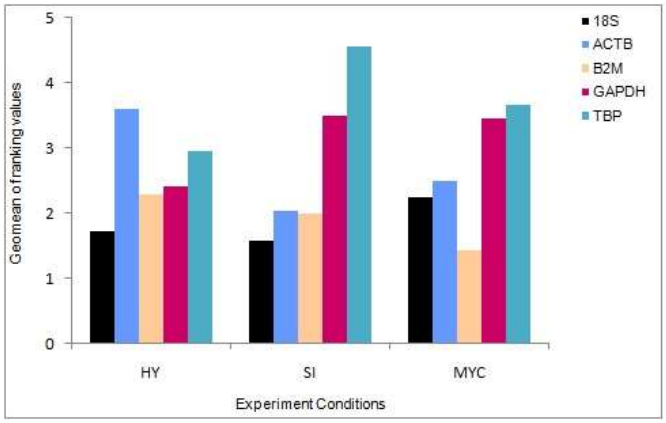

Figure 9. ***Consensus stability ranking for different experimental conditions in myeloma cells.*** *A consensus stability ranking was generated based on geometric mean of the individual ranking values obtained from four algorithms by considering three different cell lines under a specific experimental condition. HY, Hypoxic conditions; SI, Serum induction experiment; MYC, Myc inhibition experiment.*

Contrastingly, when the worst control gene used for normalization, HGF expressed high at 0.5h, then sharply decreased to near 1 fold at 1h, then retained similar expression up to 3h of the experiment followed by a decrease of near -1.5 fold in rest of SI experiment (Figure 10b).

Discussion

Gene expression analysis is crucial in molecular biology for investigation of a complex regulatory network to increase the understanding of signaling pathway, and identification of key genes involved in a biological process and as well as in

therapeutic applications (Vandesompele et al., 2002). A widely accepted method to quantify biollogicaly relevant changes in mRNA levels is qRT-PCR due to its high sensitivity, specificity, reproducibility, and reliability. However, accurate normalization is necessary for gene expression analysis using qRT-PCR since several factors including RNA quality, PCR efficiencies can affect quantification of gene expression and therefore, results might be misinterpreted (Huggett et al., 2005). Thus, various strategies have been explored for normalizing real-time PCR data including similar sample size or tissue volume, normalization of total RNA and use of reference genes. Normalization with similar sample size is relatively easy; however, the similar sample does not the same amount of RNA. Standardization to total RNA is another approach but total RNA mainly constituted by ribosomal RNA (rRNA) that rarely reflects messenger RNA (mRNA). Normalization to a reference gene is widely used for gene expression measurement in qRT-PCR as reference gene is assumed to be constantly expressed in any experimental or physical condition (Huggett et al., 2005; Caradec et al., 2010). According to recent reports, proper validation was needed for a reference gene prior to use as an internal control in qRT-PCR since the expression of reference genes varies among different cells or under different experimental/environmental conditions (Suzuki et al., 2000; Kozera & Rapacz, 2013). Again, the use of multiple reference genes is limited in gene expression quantification due to sample unavailability, costs, and labor demands. Hence, it is ideal to match sample size or volume, use of the similar quantity of RNA in reverse transcription and finally use of a stably expressed reference gene as an internal control for accurate normalization (Huggett et al., 2005).

As recent increasing of MM, the gene expression analysis is crucial to know the cellular mechanism of tumorigenesis. Moreover, an accurate normalization of qRT-PCR data is still missing for MM. Thus, the present study was designed to evaluate five common HKGs (18S, ACTB, B2M, GAPDH, and TBP) as an endogenous control for qRT-PCR normalization in MM during different experimental conditions. Here, the candidate genes were ranked based on stability values calculated by four commonly used algorithms. To reduce experimental error RNA was extracted from the same cell number, and the same amount of RNA and cDNA was used for reverse transcription and real-time PCR, respectively. Single band in an agarose gel and single peak obtained from melting curve analysis confirmed the specificity of the primer pairs. Moreover, a standard curve generated from efficacy dilutions showed a good linear relationship ($R2 > 0.99$), and their amplification efficiency ranged from 94% to 104% (Table 1) suggested the appropriateness of primers and amplification conditions

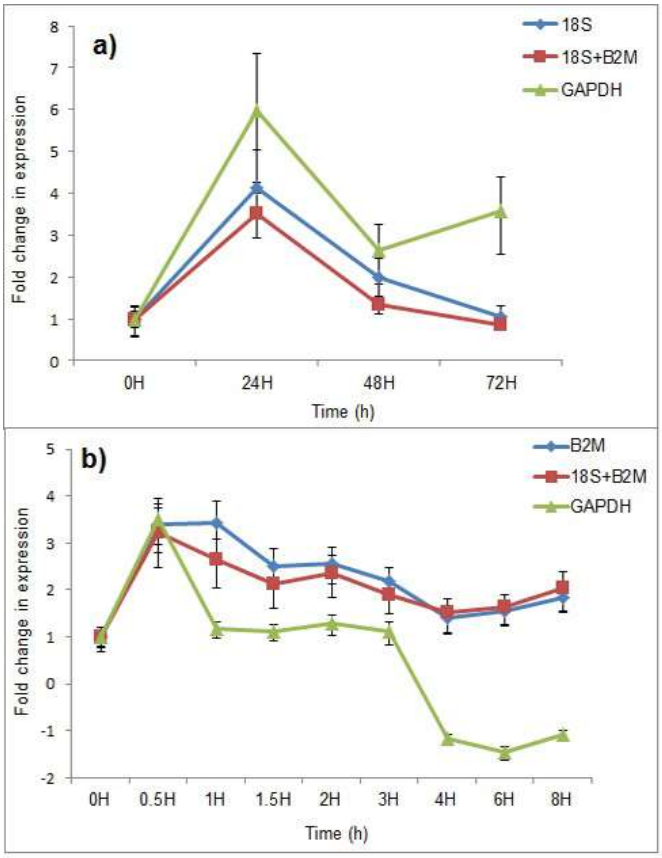

Figure 10. *Relative expression analysis of HGF using the validated reference genes. HGF expression was analysed using the best reference gene, combination of two top ranked genes and the least stable gene for normalization in qRT-PCR. The result represents as mean fold changes in relative expression when compared to first sampling stage (0h). Samples were collected from different experimental conditions (a) under hypoxia and (b) serum stimulation experiment for ANBL-6 cell lines after different time points. Bars are standard error calculated from two biological and three technical replicated.*

for the quantification of transcript abundance in qRT-PCR (Huggett et al., 2005). In addition, the mean Ct values for all the HKGs ranged from 9 to 26 indicated abundant expression of the candidate HKGs. But the variations of expression of the HKGs in all the HMCLs during different treatment (Figure 5) were corroborating that no single gene could express consistently in the cell lines under various experimental conditions.

Since hypoxia or low-oxygen concentration contributes to tumor cell survival and bone marrow (BM) microenvironment of MM patients is hypoxic, thus, hypoxia niche may consider as potential therapeutic targets (Azab et al., 2012). In addition, Cell proliferation rate is decreased when myeloma cells are culture under hypoxia compared to normoxic conditions (data not shown) which was in

accordance to a study conducted by Muz et al., where hypoxia promoted dedifferentiation while it decreased proliferation of HMCs through cell cycle arrest (Muz et al., 2014). Therefore, hypoxia can induce several biological pathways through the change in gene expression. So, hypoxia was taken into consideration to get a suitable endogenous control for qRT-PCR normalization.

18S was the optimal choice as an internal control gene across all the myeloma cells under hypoxia (Figure 9). Likewise, 18S was the most stable reference gene for comparative analysis in rat primary cultured neural cells under hypoxic conditions (Yang et al., 2008). Although, the expression of 18S was more stable than any other HKGs under hypoxic conditions for both ANBL-6 and INA-6 while GAPDH followed by 18S had a stable expression in IH-1 cells according to this study (Figure 8a). Some studies also confirmed that 18S was suitable for different normal and tumor tissues (Liu et al., 2015) although it was unreliable for some cells in hypoxia including rat brain, Hep3b and Pc3 Cells (Yang et al., 2008; Aydogan Türkoğlu & Kockar, 2012). Moreover, B2M was found the second most appropriate gene in the study (Figure 9) while it was the right choice for human chondrocytres, Hep3b and PC3 (Foldager et al., 2009; Aydogan Türkoğlu & Kockar, 2012). Our results also supported by another study conducted by Caradec et al where the expression of GAPDH, B2M, ACTB, and TBP fluctuated in various prostate and endothelial cell lines under hypoxia (Caradec et al., 2010).

For successful growth and maintains, serum was frequently used for in vitro eukaryotic cell culture. Schmittgen et al reported that the fibroblasts enter the G0 phase of the cell cycle when reducing serum concentration of the media. Thus, serum-starved fibroblasts reenter cell cycle from G0 phase through alter gene expression when adding serum to the culture (Schmittgen & Zakrajsek, 2000) and in addition, over 8600 genes change their expression in response to serum (Iyer et al., 1999). Therefore, the expression of reference genes can be fluctuated by adding serum to the serum-starved cells in the experiment. The unstable expression of GAPDH and TBP precludes their use as normalization factor in qRT-PCR for SI experiment. The comprehensive ranking recommended 18S as the most stable reference gene for IH-1 and INA-6 while B2M identified as suitable reference genes for ANBL-6 (Figure 8b). Our study consolidates 18S as the strong performer followed by B2M under SI experiment when considering three myeloma cell lines (Figure 9). These findings are in confirmation of a previous study where 18S and B2M were established as the most suitable internal control genes under serum-stimulation conditions for NIH 3T3 fibroblasts (Schmittgen & Zakrajsek, 2000).

Myc protein has been reported to activate several biological events including regulation of cell proliferation, apoptosis, and differentiation and its aberrant expression is frequently seen in multiple human cancers. Since Myc expression is crucial for myeloma cell survival, therefore, inhibition of c-Myc induces cell cycle arrest and apoptosis in myeloma cells reinforces its importance as a therapeutic target for MM (Huang et al., 2006; Holien et al., 2012). Moreover, it might possess the ability to change the expression of different genes in HMLC since it regulates about 15% of genes transcription (Holien et al., 2015). Thus, the present study was also designed to identify a stable internal reference gene during Myc inhibition experiment. On the basis of comprehensive ranking, B2M was shown to be a good candidate reference gene for ANBL-6 and IH-1 while 18S appeared to be the more stable (Figure 8c). Overall, B2M showed more stable expression followed by 18S when considering cell lines under Myc inhibition study (Figure 9).

The values of pairwise variation from GeNorm were found to be lower than cut off (0.15) in hypoxia and serum induction experiment (except for ANBL-6 under hypoxia) also suggesting that inclusion of additional gene for optimal normalization is not required (Figure 7a, 7b). Moreover, larger discrepancies were observed among the evaluated candidates when HGF gene expression normalized to the best, combination of the two best-ranked genes and the worst gene under hypoxia and SI environment (Figure 10) suggesting 18S as a reliable internal control followed by B2M for comparative analysis of gene expression in hypoxic conditions and serum-stimulation studies for MM. Although pairwise variation obtained from GeNorm suggested that these five genes are not good enough for INA-6 in qRT-PCR normalization and three genes should be used for accurate normalization in ANBL-6 and IH-1 cell lines (Figure 7c). To date, no study was performed to validate the gene expression of HKG during Myc inhibition conditions suggesting the requirement of more validation. However, a few studies employed B2M and 18S as a reference gene in qRT-PCR during Myc study (O'Donnell et al., 2006; Wang et al., 2011; Narisawa-Saito et al., 2012; Helm et al., 2013).

In conclusion, it is imperative to select appropriate reference gene in real-time PCR for gene expression analysis. Hence, five different HKG genes such as 18S, ACTB, B2M, GAPDH, and TBP were investigated on their robustness under serum stimulation, hypoxia and inhibition of the transcription factor c-Myc. With the exception of cMyc inhibition, stable expression of 18S made it a good choice as the reference gene for normalization. Moreover, B2M with some variations in the expression might also be used as a reference gene in quantitative studies. On the other hand, B2M followed by 18S are the choice of genes to use as an internal control gene in normalization of gene

expression analysis for MM during Myc inhibition conditions. Therefore, the combination of 18S and B2M might be used as reference genes to get better resolution in expression analysis for myeloma cell lines.

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