Identification and evaluation of an appropriate housekeeping gene for real time gene profiling of hepatocellular carcinoma cells cultured in three dimensional scaffold

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Abstract
Background Assessing an optimal reference gene as an internal control for target gene normalization is important during quantitative real time polymerase chain reaction (RT-qPCR) of three dimensional (3D) cell culture. Especially, gene profiling of cancer cells under a complex 3D microenvironment in a polymer scaffold provides a deeper understanding of tumor functioning in vivo.

Methods and Results Expression of six housekeeping genes (HKG’s): Glyceraldehyde-3-phosphodehydrogenase (GAPDH), β-actin (ACTB), beta-2-microglobulin (B2M), 18S ribosomal RNA (18S rRNA), peptidyl-propyl-isomerase A (PPIA), and ribosomal protein L13 (RPL-13) during two dimensional (2D) culture, and alginate-carboxymethylcellulose scaffold based 3D culture conditioned up to 21 days was analysed for hepatocellular carcinoma (Huh-7) cells. The gene expression studies were performed by determining primer efficiency, melting curve and threshold cycle analysis. Further, RT-qPCR data was validated statistically using geNorm and NormFinder softwares. The study indicated RPL-13, 18S rRNA and B2M to be stable among selected referral HKG candidates.

Conclusion An exploration of a reliable HKG is necessary for normalization of gene expression in RT-qPCR during varying cell culture conditions.

Keywords 3D culture · Hepatocellular carcinoma cell line · Housekeeping gene · Quantitative real time polymerase chain reaction

Introduction
Emerging studies indicate the significance of applying hepatocellular carcinoma (HCC) cells for recapitulating the liver tumor microenvironment in vitro using three dimensional (3D) culture. The application of 3D culture allows integration of heterogeneous cells clustered together to form a tumor spheroid model [1]. In this regard, analysing the gene expression of cells grown in 3D culture and at different time phases is significantly important for disease modeling due to its longevity. For elucidating cellular characteristics, selection of a suitable gene for normalization of target gene expression is important during quantitative real time polymerase chain reaction (RT-qPCR), known for its simplicity, specificity, and sensitivity [2]. Herein, the amplification of a target gene is monitored by quantitatively measuring the number of copies of a gene present in a sample by intercalating the amplified DNA with a fluorescent TaqMan dye. Generally, the gene expression of a target is investigated in relation to an endogenous control called housekeeping gene (HKG). The normalization of target gene is important to correct the quantity and quality differences among the culture samples [3].

In eukaryotic cellular models, application of HKG’s like ribosomal RNA based 18S rRNA, ribosomal protein L13 (RPL-13), or a gene contributing towards cytoskeleton organisation like β-actin (ACTB) are popular. Other common HKG’s include cellular metabolism enzymes like glyceraldehyde-3-phosphodehydrogenase (GAPDH) responsible for glucose breakdown, peptidyl-propyl-isomerase A (PPIA) for intracellular signalling, and an immune complex

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molecule like β-2-microglobulin (B2M) regulating tumor growth [4]. Over the years, it has been proven that GAPDH and ACTB tends to show alteration in gene expression by large variation in their mean threshold cycle (Ct) values at different experimental conditions such as hypoxia induction [5], simulated differentiation/regeneration [6], tissue specific alteration, generational gap [3], and untoward micro-RNA interference [7]. Therefore, it is indispensable to identify a reference calibrator that provides powerful insight on the relative difference in the gene expression. In 3D culture, investigating cellular growth kinetics, proliferation, and metabolism during different time intervals is important for maintenance and enrichment of spheroids. In one such instance, a study developed alginate scaffolds for human glioblastoma cell line cultured at different time intervals (7, 14, and 21 days). The influence of alginate scaffolds on cellular viability, differentiation and proliferation during different time points was profound up to 21 days [8]. Hence, longevity of 3D culture period due to generational changes in cellular metabolism and function necessitates a need for analysing appropriate HKG during different time intervals. In this study, we aimed to identify appropriate internal control during RT-qPCR using popular HKG’s like GAPDH, ACTB, 18S rRNA, PPIA, B2M, and RPL-13.

Materials and methods

Cell culture

Hepatocellular carcinoma cell line (Huh-7) (Cell repository, National Centre for Cell Science, Pune, India) was grown in DMEM complete growth media (D5796, Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (F2442, Sigma Aldrich, USA), L-Glutamine, antibiotic–antimycotic solution (P4333, Sigma Aldrich, USA) and gentamycin sulphate (G13970, Sigma Aldrich, USA) under optimum culture conditions as described previously [9]. Cells were dissociated by using 0.25% trypsin–EDTA solution (T4049, Sigma Aldrich, USA) and cell pellet was collected by centrifugation before seeding in 2D and 3D cultures maintained in a 5% CO₂ incubator.

3D cell culture

Three dimensional culture of Huh-7 was prepared using alginate-CMC cryogel based scaffold. Briefly, the cells were re-suspended in DMEM complete growth media with a final cell count of 1 × 10⁵ cells/ml. The polymer solution was prepared by dissolving alginate and CMC in 1:1 ratio with double distilled water. The prepared solution was sterilized and stored at 4 °C. Likewise, 2% calcium chloride was prepared in autoclaved water and syringe filtered (0.2 μm pore size). Using Nunc’s 35 × 10 mm cell culture dish, polymer solution was poured, and the lid was closed and tightly sealed with a parafilm (Bemis, USA). After freezing the polymer solution at −80 °C for 24 h, instant thawing with calcium chloride was performed under sterile conditions. The fabricated scaffold was washed with 1 × PBS (P3813, Sigma Aldrich, USA) prior to an incubation with cells suspended in DMEM complete growth media [1].

Nucleic acid staining

The utilized DMEM media was aspirated from 2 and 3D culture flask. The scaffold matrix was dissolved using alginate matrix dissolving solution (A1134001-Gibco, USA). Live staining of Huh-7 cells grown in 2D, and alginate-CMC based 3D cell culture was performed using acridine orange dye (A6014, Sigma Aldrich, USA). The cells were stained with 500 μl of 30 μM acridine orange stain for 15 min. The spheroids were gently washed with 1 × PBS and observed under the microscope with phase contrast and fluorescent modes of function (BX53-Olympus, Japan) [10, 11].

RNA extraction

Total RNA was extracted from Huh-7 cells grown in 2D and 3D culture at different time intervals (7, 14, and 21 days). RNA extraction was performed using TRIzol reagent (162710, Invitrogen, USA) following the manufacturer’s instructions. The quality and quantity of RNA was estimated using nanophotometer (N60-Implen, USA) [12].

Designing of primers

The primers for HKG’s were designed using RT-qPCR (TaqMan) primer designing software, GenScript (Piscataway, NJ) (https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool). The sequences for all the gene sequences were retrieved from GenBank database (www.ncbi.nlm.nih.gov). The exon boundaries in eukaryotic gene code were defined and submitted in raw format. The software provided an advantage to pick primer/probe crossing exon junction. The criteria for choosing an appropriate primer were based on a length of 18–21 bases and an amplicon size of up to 200 base pairs. Further, the obtained primer design was matched with a model organism using BLAST analysis. The gene description, primer sequences and amplicon sizes used in this study are represented in Table 1.

Quantification of the housekeeping gene

One-step RT-qPCR assay was performed with the final volume of 25 μl using PrimeScript one-step RT-qPCR kit (RR0086A, TaKaRa Biotech, Japan) as per the
manufacturer’s instructions. The reaction mixture contained 12.5 µl of 2X SYBR green master mixes, 300 nM forward and reverse primers, and 1 µl of total RNA (50 ng/µl). The reverse transcription was conditioned at a temperature of 42 °C for 5 min, and 95 °C for 10 s during one cycle followed by a PCR reaction at 95 °C for 5 s, while the annealing temperature was ranging from 55 to 64 °C for 20 s (40 cycles). A melting curve analysis was performed by heating at 95 °C for 15 s, 60 °C for 1 min and finally 95 °C for 15 s to verify the PCR product. The reaction was performed using CFX96-real time system (BioRad, USA). Based on the Ct value obtained, the expression stability of HKG’s were assessed.

The primer efficiency applied for the target amplification was evaluated in serial dilutions (1/10, 1/100, 1/1000, 1/10000, and 100,000) using triplicates. Following RT-qPCR, the Ct value obtained at RNA concentrations (0.01–100 ng/µl) was linearly plotted to determine the slope and correlation coefficient ($R^2$) [6]. After determining the corresponding values, primer efficiencies were calculated by the following equation, Eq. (1).

$$E = \left[10^{(-1/slope)} - 1\right] \times 100$$  \hspace{1cm} (1)

### Analysis of gene expression

The stability of HKG’s obtained after RT-qPCR were analysed using two softwares: NormFinder software, version 0.9, and geNorm integrated in qbaseplus, version 2.4 (Trial version). Both softwares integrated the raw Ct value obtained specifically using CFX96-real time system (BioRad, USA) in Microsoft excel format. The NormFinder software calculated the stability index by applying a statistical approach of analysis of variance (ANOVA) between the expression values of all HKG’s that were logarithmically transformed. The proportion of intra and inter group variation on overall variation was taken into consideration while deriving a normalization factor (NF). In this study, the geometric mean value of sample triplicate was applied for stability measure of candidate HKG’s during 2D and 3D culture, as different time points were considered as grouping variables [13].

Further, geNorm software for 3D culture was applied with a criterion that two ideal HKG have a minimal expression ratio across the investigated sample set regardless of cell type or treatment condition. The algorithm of the geNorm software was conditioned to calculate the standard deviation of the logarithmically transformed expression ratio across different samples for a particular gene relative to other genes under investigation. General specifications required a minimum of three reference genes and two sample types. The software analysed average stability measure (M) of each HKG and ranked them based on stepwise exclusion method. The lower the M value, higher stability was considered for the candidate HKGs [14, 15].

### Table 1 Represents the primer sequences of different genes

| Gene name       | Function                                             | Accession number | Primer code | Primer sequences (5′–3′) | Amplicon size (bp) |
|-----------------|------------------------------------------------------|------------------|-------------|--------------------------|-------------------|
| 18S rRNA        | Synthesis of ribosomal proteins                      | NC_000021.9      | 18S rRNA F  | AGAAACGGTACCACATCCA      | 121               |
|                 |                                                      |                  | 18S rRNA R  | TACAGGGCCTCGAAGAGTCC     |                   |
| PPIA            | Intracellular signalling                              | NC_000007.14     | PPIA F      | TTTCCTACGTGACTCGAACAG    | 160               |
|                 |                                                      |                  | PPIA R      | TCGATGCTCACTCGACAG       |                   |
| RPL-13          | Synthesis of ribosomal proteins                      | NC_000016.10     | RPL-13 F    | GAGCCATCGGATTTCTGGT      | 97                |
|                 |                                                      |                  | RPL-13 R    | AGTTGGACGCGTACTCTTTT     |                   |
| GAPDH           | Cellular metabolism enzyme responsible for glucose breakdown | NC_000012.12     | GAPDH F     | GGAATCAGCAAGACAGCCA      | 157               |
|                 |                                                      |                  | GAPDH R     | TCACGTCAAGGATGACCTTG     |                   |
| ACTB            | Contributes towards cytoskeleton organisation         | NC_000007.14     | ACTB F      | CCGTGAAGAAGCTAGGAG       | 106               |
|                 |                                                      |                  | ACTB R      | CACTCCAAGAGAAGAGAG       |                   |
| B2M             | Immune complex molecule                              | NC_000015.10     | B2M F       | GCGCTACTCTCTCTTTCTGG     | 133               |
|                 |                                                      |                  | B2M R       | TCCATGCCAGGAAGAAG        |                   |
| ITGB6           | Cell adhesion and signalling                          | NC_000002.12     | ITGB6 F     | TCCCGGCTTTCCAAAGAGAT     | 130               |
|                 |                                                      |                  | ITGB6 R     | TACCTACTGCAAGGGTGGGCA    |                   |
| CYP4B1          | Drug metabolism                                      | NC_000001.11     | CYP4B1 F    | CACCTGGACTTCTTGCAACAT    | 130               |
|                 |                                                      |                  | CYP4B1 R    | AGATACCATGGTGGTGTTG      |                   |
Results

Nucleic acid staining

Live imaging of Huh-7 cells in 3D culture confirmed the formation of tumor spheroids in vitro using phase contrast and fluorescent microscope. This is because acridine orange selectively binds nucleic acids by intercalation of DNA or RNA. Also, the association of acridine orange with RNA showed red fluorescence ($\lambda_{\text{excitation}} = 460$ nm blue and $\lambda_{\text{emission}} = 650$ nm), while with DNA or double-stranded RNA, acridine orange showed green fluorescence ($\lambda_{\text{excitation}} = 502$ nm blue and $\lambda_{\text{emission}} = 525$ nm) [16]. The acridine orange stained cells cultivated in 2D (Fig. 1a) showed monolayer formation, and 3D culture showed spheroid formation (Fig. 1b–d). Also, a confirmation on the influence of alginate-CMC scaffold on cellular viability of hepatocellular spheroids with respect to time was observed [1] i.e., a decrease in spheroid number linking the degradation of scaffold matrix.

Gene profiling of housekeeping genes

The melting curve analysis was performed on candidate HKG’s like GAPDH, ACTB, PPIA, RPL-13, B2M and 18S rRNA. The genetic content of HCC cells grown in 2D and 3D cultured samples showed an absolute fluorescence peak at a melting temperature of 81 °C for B2M (Fig. 2a), 88 °C for RPL-13 gene (Fig. 2b), 86 °C for 18S rRNA (Fig. 2c), 83 °C for ACTB (Fig. 2d), 87 °C for GAPDH (Fig. 2e), and 84 °C for PPIA gene (Fig. 2f) respectively. Further, amplification efficiencies of the primers were examined by deriving the slope of $C_t$ values plotted against different RNA dilutions. The efficiency of the primers obtained through the analysis was summarized with E value of approximately 94–108% (Online resource 1) and correlation coefficient $R^2 > 0.98$ (Fig. 3a). For stability analysis, the mean $C_t$ values (95% of class interval: lower–upper value) at different time intervals of culture was represented in Fig. 3b (Online resource 2). By comparing the HKGs, we found RPL-13 with a mean $C_t$ value of 25 (95% CI 24.2–25.8), 18S rRNA (14 (95% CI 12.8–15.2)), and B2M (21 (95% CI 19.9–22.1)) stable compared to GAPDH (25 (95% CI 22.8–27.2)),

Fig. 1 Phase contrast and fluorescent microscope based images of acridine orange-stained hepatocellular carcinoma cells cultured in 2D and alginate-carboxymethylcellulose based 3D culture; a 2D culture, b spheroids on day 7, c day 14, and d day 21 (×10 magnification, and 150 µm scale) 3D cultured cells. (Color figure online)
β-actin (29 (95% CI 26.2–31.8)), and PPIA (27(95% CI 25.2–28.8)).

**Stability of HKG using NormFinder and geNorm analysis**

During NormFinder analysis, RPL-13 with a stability index value of 0.033, and B2M with 0.035 were most stable compared to other genes showing a higher range of values of up to 0.15 (Fig. 3c). In this analysis, expression of both 2D and 3D culture were ranked as follows: RPL-13 > B2M > 18S rRNA > GAPDH > ACTB > PPIA (Online resource 3). While geNorm software provided a different ranking for 3D culture sample: B2M > 18S rRNA > RPL-13 > PPIA > GAPDH > ACTB (Online resource 4). According to geNorm analysis, the reference target stability M ≤ 1 was considered optimal for heterogeneous samples like treated cultured cells, biopsies of cancer, or samples of different tissues (Fig. 3d). Further, application of RPL-13 as HKG in 2D and 3D culture for β-integrin (ITGB6, role in cell adhesion and signalling) and cytochrome P450 (CYP4B1, role in drug metabolism) target normalization showed a significant upregulation up to seventh day followed by subsequent change in expression with respect to time (Fig. 3e, f).

**Discussion**

Investigating the gene functionality is of great relevance in characterisation of 3D spheroids using RT-qPCR. However, prior to performing RT-qPCR for target genes, validation of the appropriate HKG is needed for accurate normalization, which imparts precise relative quantification results. To the best of our knowledge, this is the first study to determine optimal internal control for HCC cell line cultured at different time intervals in 3D scaffold of alginate-carboxymethylcellulose polymer-based origin. A suitable selection of candidate HKG’s like GAPDH, ACTB, 18S rRNA, PPIA, RPL-13, and B2M from previous literature entailed a broader investigation. During melting curve analysis, emergence of single fluorescence peak at a particular melting temperature for 2D and 3D culture as evident in Fig. 2, indicated the presence of pure and single type of amplicon in the final product. Further, primers having an optimal efficiency were critical indicators of RT-qPCR performance, as relative gene expression of target gene normalized against a reference calibrator is calculated with $2^{-\Delta\Delta C_{t}}$ method. An acceptable range for amplification efficiency is shown in Fig. 3a. Previously conducted studies implied that achieving an approximate 100% efficiency for primer pair is necessary while applying $2^{-\Delta\Delta C_{t}}$ method. Otherwise, the
applied method is not an accurate description of fold change [17]. Hence, primer designing, and amplification efficiency are crucial for reliable RT-qPCR. However, for selecting an optimal HKG, further evaluation of Ct value was necessary. For four different sample types, the amplitude in gene expression ranges more than 3–4 cycles pointed out variability in heterogeneous cancer samples shown in Fig. 3b. Previously, a study indicated heterogeneous cancer tissues showed approximately 4 cycles of larger expression in all the HKG during raw Ct value investigation for determining the stability of a malignant tumor-like glioblastoma with non-neoplastic cells [18]. In this study, RPL-13, 18S rRNA and B2M showed stable Ct values across different culture conditions.

Earlier, without much validation, GAPDH and ACTB were the most applied HKG for target gene normalization. Many studies, however, decline the prior assumption of their expression stability remaining constant throughout a variable culture condition [6, 7]. Especially in the case of hepatocytes, breakdown of glucose and the role of the glycolytic enzyme in energy metabolism down-regulates GAPDH expression level during 3D culture [19]. Similarly, ACTB is an integral component of cellular structure and a rearrangement in 3D spheroid alters its gene expression [20]. However, expression of 18S rRNA, PPIA, RPL-13, and B2M are stably expressed among altered culture conditions of animal cell culture and have been validated in previous studies as well [4, 21]. For further analysis of the HKG’s, a robust measure using NormFinder showed the previously assumed genes to be stable among different culture conditions. However, even though geNorm analysis indicated RPL-13, 18S rRNA, and B2M to be moderately stable HKG’s for 3D culture, their ranking nature differed from NormFinder analysis due to variation in sequential normalization factors and the results were thus considered trivial. Among the two approaches, the NormFinder follows a descriptive statistical
approach for HKG selection compared to geNorm. Also, NormFinder considers both inter and intra group variation to evaluate stability while geNorm determines pairwise standard deviation of Ct values of all genes excluding the HKG’s with the lowest stability [22, 23]. Nonetheless, the suitability of statistical method differs based on experimental settings. However, there is no consensus on the statistical approach to select the best HKG nor on its application for RT-qPCR data normalization. Since the PCR evolution, the application of a single HKG that is most stable for normalization is conventionally acceptable. However, the implication of averaging the Ct values of two or more HKG for the normalization of target genes provides superior accuracy.

**Conclusion**

In the current study, validation of an appropriate housekeeping gene was performed for liver cancer cell line cultured in different time intervals of 3D culture. For elucidating the natural cellular mechanisms, analysing the gene expression is one of the main criteria. In this aspect, choosing the best HKG for target gene normalization is considerably advantageous in obtaining accurate results. During variable culture conditions like hypoxia-normoxia, time-dependent changes, and drug dose-dependent studies in 3D culture, we recommend RPL-13, 18S rRNA and B2M for gene normalization during RT-qPCR analysis. Also, the implication of averaging the Ct values of best HKG’s (two or more instead of one) is a good practise to avoid variation in the outcome of target gene expression. However, the stability of the HKG applied in this study may vary for other cell types or culture conditions due to their genetic heterogeneity. Hence, this study can be extended in the future for other cell lines with novel 3D culture techniques for accurate RT-qPCR analysis.

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**Author contributions** AKB: Writing and original draft preparation. SK: Conceptualization, writing and editing of the manuscript. PR: Editing and proofreading of the manuscript.

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**Data availability** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Declarations**

**Conflict of interest** AKB, SK and PR have no conflict of interest.

**Research involving human and animals rights** This article doesn’t contain any studies with human participants or animals performed by any of the authors.

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