Improvement of Gene Expression Studies in the DimethylNitrosamine Induced Liver Fibrosis Model in the Rat Using Selected Reference Genes for Quantitative Real Time-PCR Analysis

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Abstract

**Background:** Liver fibrosis is a reaction to chronic liver injury characterized by excessive accumulation of collagen. Due to their importance as biomarkers, the changes in gene expression in the liver during the development of fibrosis and its subsequent outcomes of cirrhosis, neoplasia or resolution are intensely studied. Quantitative real-time PCR (qPCR) with its ability to detect and measure minute amounts of nucleic acids have been increasingly used in these studies. In qPCR, the quantitation of mRNA is relative and the accuracy of results dependent on the reference genes used for standardization. However, many genes studied are normalized against single reference genes, usually housekeeping genes, without adequate justification.

**Methods:** For the dimethylnitrosamine (DMN) induced liver fibrosis rat model, we tested 8 commonly used candidate genes (Actb, Alb, Sdhb, B2m, Rn18s, Hprt1, Ppia and Gapdh) to determine their suitability as reference genes. qPCR results were analysed using four commonly used programs; NormFinder, GeNorm, Comparative ΔCt methods and BestKeeper.

**Result:** It was determined that Gapdh and B2m were the most stable genes in normal liver. However, in DMN treated livers, Gapdh and Ppia were the most stably expressed reference genes. We validated these reference genes by using them to normalize the expression of four genes; Tgfb 1, Cor1 1, Col3a1 and Tnf known to be highly expressed in liver fibrosis.

**Conclusion:** Gapdh and Ppia are the most suitable reference genes for the normalization of qPCR data in gene expression studies of the liver in the DMN induced liver fibrosis model in the rat. We advise against the use of Actb in this experimental setting because of its low expression stability.

**Keywords:** DMN; Liver fibrosis; Gene expression; Reference gene; qPCR

Introduction

Fibrosis of the liver is due to the progressive accumulation of connective tissue caused by various etiologies. The most common causes being infection with hepatitis B or C virus, metabolic disorders, alcohol abuse and autoimmune diseases [1]. Fibrosis can lead to cirrhosis, hepatocellular cancer and eventual liver failure [2]. In diseased liver, deposition of the extracellular matrix is mainly due to activated hepatic stellate cells that differentiate into myofibroblasts. These cells also stimulate the inflammatory response and interfere with the normal resorption of ECM [1]. Besides myofibroblasts, different populations of macrophages have also been identified to play critical roles throughout the initiation, maintenance and resolution of liver fibrosis [3]. Thus in this scenario of complex tissue changes and cellular interactions, it is important to measure altered gene expression in a reliable and accurate manner.

Quantitative real-time PCR (qPCR) is a primary tool for studying changes in relative gene expression in different tissues and experimental conditions [7]. Although these genes are considered to be constitutively expressed, there is usually variation in the level of expression depending on tissue type as well as physiological, pathological and experimental situations. These variations in the reference gene expression, will lead to misinterpretation of data. Thus identification and validation of suitable reference genes for a specific model is crucial to the accuracy of the gene expression pattern [8,9]. In preclinical studies, many groups have used animal models as part of experimental liver fibrosis research. The different rodent animal models used and the translational aspects were recently reviewed by Liedtke et al. [10]. For rats, these include models made using bile duct ligation, carbon tetrachloride, gene knock-outs and DMN. The DMN model has many features of human liver fibrosis [11–13] and is the one that our group currently uses to test potential antifibrotic compounds. In this model, liver fibrosis is induced by intra-peritoneal injections of DMN for 3 consecutive days a week, for a total of 4 weeks. At the end of the 4th week of DMN administration, animals have typically developed severe fibrosis [14,15]. We recognise that for
accurate documentation of gene expression in each animal model studied, the reference genes must first be determined.

We reviewed 33 articles [11,12,14,16-45] to identify genes that were used for normalizing gene expression in studies where DMN was used to induce liver fibrosis in the rat. The most commonly used reference genes are Glyceraldehyde-3-phosphate dehydrogenase (Gapdh; 17 times), beta-Actin (Actb; 7 times), 18S ribosomal RNA (Rn18s; 5 times), Cyclophilin A (Ppia; 3 times) and Hypoxanthine phosphoribosyltransferase 1 (Hprt1; 2 times). In addition, we included three other commonly used housekeeping genes for normalizing gene expression in the rat; b-2-microglobulin (B2m), Succinate Dehydrogenase (Sdha) and Albumin (Alb) [46-49]. The 8 selected genes were evaluated using the mathematical algorithms; GeNorm [50], NormFinder [51], Comparative ΔCt method [52] and BestKeeper [53] to determine which of them was the most stable reference gene for this liver fibrosis model.

Materials and Methods

Animals

Male Wistar rats, 4 weeks old, were purchased from the National University of Singapore, Singapore and acclimatised for a week. All animals were maintained at room temperature of 22 ± 1°C, with 12 h light and 12 h dark cycles with access to food and water ad libitum. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC No 201007-28); Temasek Polytechnic, Singapore and all experiments were performed in accordance with the approved guidelines and regulations.

Induction and assessment of liver fibrosis

Liver fibrosis was induced by intra peritoneal (i.p.) injections of dimethylnitrosamine (DMN) into rats (n=5) at a dose of 10 mg/kg body weight. Injections were carried out for 3 consecutive days each week over a 4 week period. Control rats (n=5) were injected with 0.9% physiological saline. Blood was collected three times from the tail vein of all animals for serum biochemical analysis of alanine aminotransferase enzyme (ALT). The first collection was done prior to DMN administration, whilst the second collection was done after 2 weeks of DMN treatment. At the end of the 4th week, the third blood collection was performed and the rats were euthanized. Complete post-mortem examination was performed. Portions of liver were collected and stored in –80°C for gene expression analysis and fixed in buffered formalin for histopathological analysis. 5 µm thick paraffin sections stained with Masson trichrome [54,55] were examined by a pathologist and the degree of fibrosis determined using the Ishak score [56]. In the Ishak score, the severity of fibrosis is ranked from 0 to 6, with 0 corresponding to no fibrosis and 6 indicating the most severe fibrosis, also referred to as cirrhosis. In summary; 0: No fibrosis, 1: Fibrous expansion of some portal areas, with or without short fibrous septa, 2: Fibrous expansion of most portal areas, with or without short fibrous septa, 3: Fibrous expansion of most portal areas, occasional portal to portal (P-P) bridging, 4: Fibrous expansion of portal areas with marked bridging (portal to portal (P-P) as well as portal to central (P-C)), 5: Marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis), 6: Cirrhosis, probable or definite.

RNA extraction and cDNA synthesis

The liver specimens were homogenised and total RNA was extracted using miRNeasy Mini kit (Qiagen) according to the instructions of the manufacturer. The RNA concentration and purity was determined using a nanophotometer (Implen GmbH). RNA samples with 260:280 ratio from 1.9 to 2.1 were used for further analysis. RNA samples were stored in –80°C freezer for long term storage or stored in ice for immediate cDNA synthesis. For cDNA synthesis, 2 µg of total RNA was reverse transcribed using High Capacity RNA-to-cDNA kit (life technologies). A 20 µL reaction mixture containing 1 µL of 20X RT Enzyme mix, 10 µL of 2X RT Buffer, 2 µg of RNA sample and nuclease free water was added. The reaction mixture was incubated for 60 min at 37°C and the reaction was terminated by heating to 95°C for 5 min. The cDNA was stored at –20°C until the RT-PCR experiments.

Reference gene selection and primer design

We searched the literature for articles on gene expression where DMN was used to induce liver fibrosis in the rat. We also scanned the literature for other commonly used housekeeping genes in the rat. A list of eight most commonly used reference genes was identified. They were 18S Ribosomal protein (Rn18s), Albumin (Alb), b-2-microglobulin (B2m), Cyclophilin A (Ppia), Glyceraldehyde-3-phosphate dehydrogenase (Gapdh), Hypoxanthine phosphoribosyltransferase1 (Hprt1), Succinate dehydrogenase (Sdha) and beta Actin (Actb). The primer for Actb was designed based on available sequences using the Primer3 software. Primers for Alb, B2m, Ppia, Gapdh, Hprt1, Rn18s and Sdha were obtained from the literature [46,57]. The primers are listed in Table 1.

| Gene Symbol | Gene Name | Gene Function | Primer | Product Size (bp) | Efficiency (%) | Correlation Coefficient (R²) |
|-------------|-----------|---------------|--------|------------------|----------------|-----------------------------|
| Rn18s       | 18S Ribosomal RNA | Protein Synthesis | F: GTAACCGGTGGAAACCCATT R: CGCTACTACCGATTGGATGG | 151 | 90.4 | 0.996 |
| Alb         | Albumin   | Major plasma protein | F: GATGCGGTGAAGAGAAGAAC R: CGTGACGGCATTCTTGTG | 196 | 81.5 | 0.997 |
| B2m         | Beta 2 Microglobulin | Beta-chain of major histocompatibility complex | F: ACATCCTGGCTACCTGAA R: AGTGTCGTTGCCAGGTTG | 109 | 92.6 | 0.998 |
| Ppia        | Cyclophilin A | Serine-threonine phosphatase inhibitor | F: AGCAGCTGGGAGAAAGAGT R: GGACCTCTCATTTGTCAG | 248 | 90.6 | 0.995 |
| Gapdh       | Glyceraldehyde-3-Phosphate Dehydrogenase | Glycolysis pathway enzyme | F: GTATCGGAGCGCCTGTTTTAC R: CCTGCCTGGGTGGTAGAGGTAC | 128 | 92.1 | 0.995 |
| Hprt1       | Hypoxanthine Phosphoribosyltransferase 1 | Metabolic salvage of purines | F: GCTAAGATTGGTGAAGAGGT R: AATTAGCAAGGGTACAGAAG | 157 | 93.8 | 0.994 |
| Sdha        | Succinate Dehydrogenase | TCA pathway enzyme | F: AGACTTGTAGGGCTAGGGAATG R: TCATCAATCCGCACCTTGTA | 160 | 93.4 | 0.998 |
| Actb        | Beta Actin | Cytoskeletal structural protein | F: AGCCATGTAGTACGACATCC R: CTGCTACGTGTGTTGAAA | 228 | 108.2 | 0.995 |

Table 1: Reference gene primers: Primer information for the eight candidate reference genes.
Quantitative real-time PCR

The PCR reactions were performed on a Rotor Gene Q machine (Qiagen). The reactions were run in triplicates with 2 μL of primer pairs, 5 μL of SYBR green master mix, 1 μL of RNA-free water and 2 μL of cDNA template. The PCR was carried out with the following conditions: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing and extension together at 60°C for 45 s. After the last cycle the melting curve was determined in the range 60–95°C. Negative control samples were always included in the amplification reactions to check for contamination. Specificity of amplification was confirmed by melting curve analysis.

Determination of reference gene expression stability

All cDNA samples were normalized at the RNA level. Raw qRT-PCR amplification data were exported from Rotor Gene Q Series Software 2.0.2 (Qiagen) to Microsoft Excel. The software LinRegPCR [58] was used to calculate the efficiencies for all the reactions separately. LinRegPCR is a free software tool that uses non-baseline-corrected data to perform a baseline correction on each sample separately, then determine a window-of-linearity and then uses linear regression analysis to fit a straight line through the PCR data set. From the slope of this line the PCR efficiency of each individual sample is calculated. The efficiency corrected Ct-values were used in BestKeeper, NormFinder, GeNorm and RefFinder (comparative ΔCt method) to rank the stabilities of the candidate reference genes firstly in livers from control and secondly in livers from both control and DMN treated animals. Besides the stability ranking made by each of the four algorithms, recommended comprehensive stability rankings of the candidate genes in control and control plus DMN treated rats were generated using RefFinder [59].

Validation of reference genes

The expression pattern of four genes, Tgfb 1, Col1a1, Col3a1 and Tnf were analysed using different normalisation strategies. This included applying the most stable reference genes identified from the above software, singly or in combination, to the gene expression data using REST 2009 software (Qiagen) [60]. In addition, the commonly used housekeeping gene, Actb, which was determined to be the least stable, was also tested on the same data set to evaluate the expression pattern of the four genes. The primers for the above target genes are shown in Table 2.

Results

Assessment of liver fibrosis

ALT levels were significantly elevated after 2 and 4 weeks of DMN administration (Figure 1). At sacrifice, after 4 weeks of DMN administration, the average fibrosis score for DMN treated rats was 5 out of a maximum of 6 based on the Ishak score (Table 3) [56]. There was marked bridging of portal to portal areas with occasional nodule formation (a). Collagen strands (stained blue) are present in between hepatic cords and separating hepatocytes (b). Liver section from a normal control rat, (c) 40X magnification.

Table 2: Primer set for target genes: Primer information for the selected target genes.

| Primer | Primer Sequence 5’→3’ | Product Size (bp) |
|--------|----------------------|-------------------|
| Tgfb 1 | F: ATACGCGCTGAGTGGCTGTCT R: TGGGACTGATCCACCATGATT | 153 |
| Col1a1 | F: GAGAGCATGACCGATGGATT R: CATCTTTTCCAGGAGGTCCA | 173 |
| Col3a1 | F: GTCCACGAGGTGACAAAGGT R: CCCATTTGGGAACTTCTCCT | 189 |
| Tnf | F: TGATCCGAGAGTGGAGAAGT G: CCCATTTGGGAACTCCTCTT | 185 |

Table 3: Severity of fibrosis in the liver represented as fibrosis score.

| Fibrosis score (Using Ishak Score) |
|-----------------------------------|
| DMN (n=5) | Control (n=5) |
| 5.2 ± 0.8 | 0 ± 0.0 |

Figure 1: Assessment of liver damage by serum alanine aminotransferase (ALT) level. Serum alanine aminotransferase (ALT) of DMN treated rats at weeks 0, 2 and 4 after the last DMN injection. The data are represented as the means ± SD (n=6-8). *P<0.05 compared with normal control group. ALT levels in DMN treated rats were significantly elevated after 2 and 4 weeks of treatment when compared to control rats.

Figure 2: Evaluation of liver fibrosis by histopathological examination. Photomicrographs of Masson’s Trichrome stained sections of livers from rats. Liver section from a rat after receiving 4 weeks of DMN, (a) 40X magnification, and (b) 100X magnification. There is fibrous expansion of portal areas with marked portal to portal as well as portal to central bridging, with nodular formation (a). Collagen strands (stained blue) are present in between hepatic cords and separating hepatocytes (b). Liver section from a normal control rat, (c) 40X magnification.
formation (Figure 2a). At higher magnification, collagen strands were visible between hepatic cells, some of which were necrotic (Figure 2b). The fibrosis score for control rats was 0 (Figure 2c).

Amplification efficiency

The amplification efficiency for all qRT-PCR reactions was calculated using the LinRegPCR software. The results were included in Table 1. The PCR amplification efficiency for each reference gene ranged from 90.2 to 108.2%, with the exception of Alb which showed an efficiency of 81.5%. The amplification performance of each primer was verified by RT-PCR and primer specificity was tested by melting curve analysis. The linear regression coefficient for all candidate reference genes ranged between 0.994 and 0.998.

Gene expression levels of candidate reference genes

The expression level of all reference genes were calculated using threshold cycle (Ct) values for two groups; control and DMN treated rats. The transcription differences were shown in the box plot of Ct values (Figure 3a) for control rats and Figure 3b for DMN treated rats. For the control group, the SD for Ct values of all genes were within a narrow range, 0.46 to 0.87. For DMN treated rats, the SD range was wider, ranging from 1.09 to 1.85 for all genes except 18s (Table 4).

Analysis of reference gene stability using NormFinder, GeNorm, Comparative ΔCt method and BestKeeper. The Ct data from the two groups; control and control plus DMN treated rats were analysed, using four different algorithms. NormFinder calculates the stability value for each reference gene and inter and intragroup variation between treated and untreated samples. The candidate gene with the lowest intergroup variation combined with the lowest average intragroup variation and the lowest stability value would be the most stable housekeeping gene. For control rats, NormFinder analysis showed that Gapdh and B2m with stability values (S) of 0.19 and 0.2 (Figure 4a) were the most stable reference genes. However, when data from the control plus DMN treated rats, were analysed, Gapdh and Ppia with S values of 0.81 and 0.83, respectively were the most stable reference genes (Figure 5a). Actb and Alb were found to be the least stable genes for both groups.

GeNorm algorithm calculates the stability measure (M) for a reference gene. Stable expression is indicated by lower values of M. The ranking for the reference genes were similar for control and control plus DMN treated groups (Figures 4b and 5b). Gapdh and Ppia were the most stable housekeeping genes with identical M values in both groups. Actb and Alb were found to be the least stable genes with M. However, the M values for each reference gene in the control group were lower in the control group than the control plus DMN treated group. GeNorm was also used to determine the optimal number of reference genes required for normalisation. This was done through calculating the pairwise variation (Vn/Vn+1) between sequential pairs of candidate reference genes. The value, 0.15 was adopted as the cut-off value to determine the optimal number of reference genes for normalizing the expression of genes of interest. According to GeNorm, three reference genes (Figure 6) would be the optimal number to use for normalization of the current gene expression data.

The Comparative ΔCt method determines the most stable reference gene by comparing the Ct ratio or relative expression of “pair of genes”

![Figure 3: Expression profile of 8 candidate reference genes in rat livers represented as box plots of qPCR (Ct) values. Box plots normal livers; n=5 (a) and DMN treated livers; n=5 (b). The horizontal line within each box represents the median Ct value and separates the upper and lower quartiles. Vertical lines indicate the range of values. There was lower variation in Ct values of reference genes in control livers compared to those from DMN treated livers.](image)

| Gene | Control Average ± SD | DMN Average ± SD |
|------|----------------------|------------------|
| Rn18s | 26.41 ± 0.64         | 26.19 ± 0.57     |
| Alb  | 23.36 ± 0.49         | 22.43 ± 1.09     |
| B2m  | 23.47 ± 0.46         | 24.95 ± 1.60     |
| Ppia | 23.79 ± 0.47         | 25.17 ± 1.49     |
| Gapdh| 26.50 ± 0.50         | 27.93 ± 1.51     |
| Hpirt| 26.04 ± 0.61         | 27.28 ± 1.52     |
| Sdha | 22.57 ± 0.57         | 24.31 ± 1.85     |
| Actb | 15.70 ± 0.87         | 14.69 ± 1.25     |

Table 4: Average RT-qPCR threshold (Ct) values of 8 reference genes in liver of control and control plus DMN treated rats. Average standard deviation (SD) was calculated from 5 animals in each group, with 3 replicates from each sample (n=15).
Figure 4: Expression stability of 8 candidate reference genes in normal (control) livers calculated by 4 algorithms. (a) NormFinder (b) GeNorm (c) Comparative ΔCt method and (d) BestKeeper. For all algorithms, lower values indicate more stable genes. The genes with the lowest values are most suitable as reference genes.

Figure 5: Expression stability of 8 candidate reference genes in all livers (normal and DMN treated) calculated by 4 algorithms. (a) NormFinder (b) GeNorm (c) Comparative ΔCt method and (d) BestKeeper. For all algorithms, lower values indicate more stable genes. The gene with the lowest value is the most suitable reference gene whilst the gene with the highest value is the most unsuitable reference gene.
within each sample and determines the standard deviation of ΔCt. The gene with the highest standard deviation of ΔCt is the least stable gene and vice versa. In the control group, Gapdh and B2m were the most stable genes (Figure 4c). However, in the control plus DMN treated group, Gapdh and Ppia were identified as the most stable genes. For both groups, Actb was the least stable gene.

The Microsoft Excel based tool BestKeeper uses raw Ct values as input to determine the stability of reference gene expression. BestKeeper calculates the expression variation based on standard deviation (SD) and Pearson correlation coefficient for each reference gene pair. The program establishes the BestKeeper index which is the geometric mean based on raw Ct values by pairwise correlation analysis for each reference gene pair. The most stable reference genes predicted by this program were different from the other three programs used. For the control group, the most stable genes were Gapdh and B2m whereas the least stable genes were Rn18s and Actb (Figure 4d). For the control plus DMN treated group, this program indicated that Rn18s and Ppia were the most stable genes whereas the least stable genes were B2m and Sdha (Figure 5d).

The Ct values were further analysed using RefFinder and the recommended comprehensive ranking for the most stable reference genes were Gapdh and B2m for the control group. The least stable genes for this group were Hprt1 and Actb whereas the least stable genes were Gapdh and Ppia whereas the least stable genes were Ppia and B2m (Table 5a). For the control plus DMN treated group, the most stable genes were Gapdh and Ppia whereas the least stable genes were Sdha and Actb (Table 5b).
Validation of reference genes

To show the effect of different reference genes on the outcome of an actual experiment, we evaluated the expression pattern of four genes, Tgfb1, Col1a1, Col3a1 and Tnf, in our current experiment, using rat liver tissue after 4 weeks of DMN treatment. Tgfb1, Col1a1, Col3a1 and Tnf were significantly over-expressed when normalised using the most stable reference gene, Gapdh as well as the combinations of 2 (Gapdh and Ppia) and 3 most stable genes (Gapdh, Ppia and Rn18s). When the least stable gene Actb was used as the normalisation reference gene, the expression of Tgfb1 and Col1a1 was low. There was no increase in the expression of Col3a1 and Tnf expression was down-regulated (Figures 6 and 7).

Discussion

qPCR is a powerful method that combines high specificity and sensitivity for studying mRNA expression level in tissues or cell culture. However, to obtain accurate data, suitable reference gene(s) must be used for normalization of qPCR results [4,5] to ensure that the expression profile of the target gene is not misrepresented. Increasing awareness of the importance of selection of suitable reference genes in preclinical research has led to recent work to identify the least variable housekeeping genes in 11 tissue types in normal rats [49]. In that study nearly two thirds of the 48 mRNA targets showed relatively low expression variability and were considered to be potential reference genes. For normal liver, B2m was identified to be a suitable reference gene.

We have similarly identified B2m to be a suitable reference gene for normal liver, though our results show that it is not the most, but the second most stable gene; after Gapdh.

When the liver is treated with DMN for 4 weeks, the most stable gene remains as Gapdh, but the second most stable is now Ppia. The comprehensive ranking of data from four different algorithms showed that for DMN treated liver, B2m was ranked fifth out of eight most stable genes and would not be suitable as a reference gene for this experimental setting. We caution that the most stable genes in normal tissues may not be the same when subjected to experimentation.

From the results of our experiment and from the data presented in the literature, we realise that it is extremely important to state clearly the experimental conditions and tissue types that are being studied using a particular set of reference genes. Svingen et al. used NormFinder to study gene expression data from selected control rat tissues. They analysed data collectively from six tissue types (liver, adrenal, prostate, fat pad, testis and ovaries) of juvenile rats and four tissue types (liver, prostate, fat pad and testis) from adult rats. Out of a total of 12 reference genes tested, they identified Hprt and Sdha as the two most stable reference genes [61]. When they included data from juvenile and adult rats exposed to chemical mixtures in the analysis, Hprt and Sdha remained the most stable genes although they reported the lower ranked genes became more unstable. Thus for their experimental setting, the reference genes for normal and treated tissues were the same.

However, within the same experiment, it should not be assumed that the most stable reference genes determined for the experimental setting and overall group of tissues studied will be the most suitable genes for the study of subsets of tissues from that same experimental setting. In the same study by Svingen et al. the most and least stable reference genes were different when different combinations of normal juvenile tissues were studied. For example, when liver and fat pad were studied together, B2m and Tbp were the most and least stable genes. However, when prostate and fat pad were studied together, Rps29 and Actb were the most and least stable genes. Thus their data showed that Hprt could be used as the most suitable reference gene when they compared gene expression across all the different tissue types and chemical treatments in their study. However, if they were to compare...
gene expression amongst two specific tissue types, their results would be more accurate if they used the most stable reference gene for those two tissue types and would be highly erroneous if they used Hprt. Hence, it is also important to determine the most stable reference genes for subsets of tissues that are being studied within the overall experimental setting.

All studies on gene expression using the rat DMN model of liver fibrosis reported so far, have used a single non validated endogenous reference gene for normalization of gene expression data [11,12,45,62]. The present study is the first to determine suitable reference genes for the DMN model of liver fibrosis in the rat. The development of liver fibrosis in this present experiment was verified by the significantly elevated ALT levels and the fibrosis scores on histopathological examination.

The expression stabilities of eight potential reference genes (Actb, Rn18s, Alb, B2m, Ppia, Gapdh, Hprt1 and Sdha) were analysed using four widely recognized programs (GeNorm, NormFinder, Comparative ΔCt method and BestKeeper). GeNorm uses two parameters; M (average expression stability) and V (pairwise variation) to quantify reference gene expression stability. A low M value indicates a more stable expression, hence, increasing the suitability of a particular gene as a reference gene. Other advantages of GeNorm are that it is minimally affected by expression intensity of the candidate genes, does not require a normal distribution of data and, since the approach is based on multiple pair-wise comparisons; a large sample size is not required [50]. The NormFinder algorithm ranks candidate reference genes according to the least estimated intra and inter group variation. NormFinder aims to identify candidate reference genes(s) with an inter group variation as close to zero as possible, while at the same time having small intra-group variation. However, NormFinder is biased towards candidate reference genes that have overall similar Ct values and the larger the sample size, the more robust the algorithm becomes [51].

The Comparative ΔCt method assesses the most stable RGs by comparing the relative expression of “pairs of genes” within each tissue sample or each treatment, and determines the standard deviation of ΔCt. The gene with the highest standard deviation of ΔCt is the least stable gene and vice versa [52]. Unlike GeNorm, NormFinder and Comparative ΔCt methods, input data for analysis by BestKeeper was raw Ct values of each gene. The overall stability in gene expression is shown by calculated variations in standard deviation (SD), coefficient of correlation (r) and percentage covariance (CV). The lowest SD value indicates the most stable RG expression [53].

Despite the different mathematical algorithms used by each program, three of these programs, NormFinder, GeNorm and Comparative ΔCt method determined that Gapdh and Ppia were the most suitable reference genes (Table 4). However, the fourth program, BestKeeper identified two other genes; Rn18s and Alb as the most suitable reference genes. The results from GeNorm, NormFinder and the Comparative ΔCt method were more consistent with each other than with the BestKeeper method. This discordance in ranking by the BestKeeper method from the other three algorithms was similarly made by Chen et al. [63] in their study on gene expression in human umbilical vein endothelial cells.

Both Gapdh and Ppia are genes which control basic, though different cellular functions. Gapdh encodes an enzyme that plays an important role in energy metabolism and production of ATP for glycolysis in the cytoplasm [64]. Ppia encodes a protein which regulates protein folding and trafficking [65]. Our findings that they are the most stable reference genes, suggest that they are not affected in liver cells which undergo changes that result in tissue fibrosis. This was not the case for Actb. Though considered a housekeeping gene leading to its frequent use for normalisation in many types of gene expression studies, including the rat DMN model [12], our study found that Actb was the least stable reference gene. This could be due to the function of the Actb gene which encodes for proteins responsible for cell structure and integrity. Thus we caution against the use of Actb in this experimental model.

In the present study, the selected reference genes were validated by using them in turn as reference genes to determine the expression of target genes known to be up-regulated in liver fibrosis. These target genes were a) Tgfb1, which acts as a central regulator of cell growth and differentiation [66,67], b) Tnf, a cytokine involved in acute phase reactions [68,69] and c) Col1a1 and Col3a1, which encode for types I and III pro-collagen respectively [1,3,70].

The most and least stable reference genes and the combination of 2 and 3 most stable reference genes were used for expression normalization. The expression level for the target genes were highest when normalized against the single most stable reference gene (Gapdh) followed by the combinations of 2 (Gapdh and Ppia) and then 3 (Gapdh, Ppia and Rn18s) reference genes. The least stable reference gene (Actb) had the lowest levels of target gene expression.

The use of more than one reference gene normalization can improve the reliability of gene expression studies [71]. The common practice of using one non validated reference gene can lead to erroneous results, as shown in our case, for Actb. One reference gene is suitable to determine “on/off” expression or huge expression differences. This was also true for our case, with Gapdh. According to GeNorm pairwise analysis, the combination of 3 most stable reference genes (Gapdh, Ppia and Rn18s) were recommended for our data set. However, the target genes’ expression levels were higher when normalized against the two most stable genes than against the recommended three most stable genes. Thus, in the DMN model, we believe that two reference genes would be sufficient to determine differences in expression levels of the intended target genes. According to Vandesompele et al. three reference genes could be used to measure small expression differences more reliably [72].

Conclusion

In conclusion, we used qPCR to obtain data on gene expression patterns of eight candidate reference genes in the liver in the DMN induced model of liver fibrosis in the rat. Analysis of these data by NormFinder, GeNorm, Comparative ΔCt Method and BestKeeper programs determined that Gapdh and Ppia were the 2 most suitable reference genes for normalisation in the liver in the rat DMN model of liver fibrosis. The use of these genes as an internal reference gene pair will greatly enhance the reliability and robustness of gene expression data for this experimental model.

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