Validation of Housekeeping gene expression levels in normal, colorectal carcinoma and liver metastases in tissue

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ABSTRACT

In this study, the expression level of two housekeeping genes, 18S ribosomal RNA and β-glucuronidase (GUS), were quantified in two sets of patient samples (A1 and p222) consisting of normal, colorectal carcinoma and liver metastasis using real-time quantitative RT-PCR. A third housekeeping gene, porphobilinogen deaminase (PBGD) was optimized. Results showed that there was no significant modulation of gene expression levels of 18S rRNA and GUS mRNA in normal, colorectal carcinoma and liver metastases of samples (P-value > 0.05). Thus, 18S and GUS are suitable internal controls for gene expression analysis under these experimental conditions. Previous results of GAPDH were also analyzed and showed significant upregulation in patient sample (p235) (P<0.001). Limitations to the use of these HKGs are discussed and the solutions to overcome these are proposed.

INTRODUCTION

Real time-PCR (RT-PCR) is increasingly being used to quantitate mRNA due to its large dynamic range and sufficiency of small sample amount. However, there is inherent variability in the technique that must be corrected for. This includes the amount of RNA degradation, random sample-to-sample variation, RNAse or RNA/DNA contamination, enzymatic efficiency, differences in initial template amount, differences in overall transcriptional activity between cells and carry-forward contamination from RNA extraction. These variation compromise the quantity and quality of RNA used as template in RT-PCR. Furthermore, small errors in initial template amount between tubes will be magnified in the amplification steps thus leading to spurious results. In order for results to be reliable at all, the use of standards is necessary.

Internal standards involve the use of a housekeeping gene (HKG) that is already present in the sample. The term “housekeeping gene” implies that it has an important role to play in maintaining the essential day-to-day functions in the cell. Hence, it is generally assumed that HKGs are constantly expressed in all tissues in the body. However, it has been discovered that many housekeeping genes such as GAPDH and B-actin, modulate in response to certain experimental treatments and even between cell types and cells at different stages. (2,3,5). The most important requirement for a HKG is the maintenance of a stable and consistent expression across the samples used under the experimental conditions of the study.

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Three housekeeping genes, 18S ribosomal RNA, porphobilinogen deaminase (PBGD) and β-glucuronidase (GUS), were selected in this study to determine their potential for use in future quantitative gene expression analysis for normal, colorectal tumour and metastatic cells used in our lab. Stability of the expression of these three HKGs was investigated in patient clinical samples using real-time quantitative RT-PCR.

MATERIALS AND METHODS

(i) Primer design

Primers for β-glucuronidase (GUS) and porphobilinogen deaminase (PBGD) were designed using the LightCycler Probe Design Software (Roche 2001). Primer sequences for 18S ribosomal RNA were obtained from a previous study in a published journal (3). All primers were purchased from Research Biolabs and used according to manufacturer’s instructions (RB-TAGC).

(ii) Optimization

Conditions for each primer pair for were optimized for maximum efficiency and specificity by varying the annealing temperature, Mg2+ concentration and primer concentration in a 10 µL reaction mix. This was carried out according to manufacturer’s specification in the Instruction Manual Version 3 (Cat. No. 2 015137). For optimization purposes, HCT116 cell line was used. The reagents were obtained from the LightCycler RNA Amplification Kit and DNA Master SYBR Green supplied by Roche. Reaction mix was added into glass capillaries and centrifuged in pre-cooled centrifuge adapters at 2000 rpm, 4°C for 10 s. PCR products were collected into Ependorf tubes by centrifuging at 2000 rpm for 10 s. Products were run on a 2% agarose gel using electrophoresis to check for specificity of reaction and confirm the presence of the desired product.

(iii) Standard curve formation

A standard curve was constructed individually for each HKG. 4 µl of RNA extract of four different dilutions of HCT116 ranging from 25ng/µL to 0.025ng/µL was added in duplicates to 6 µL of master mix as specified by the manufacturer. A water control was also set up with 4 µL H2O in place of RNA. The LightCycler Data Analysis software draws the best fit line for the log of RNA concentration plotted against their crossing points or cycle threshold values (C_T). The C_T value is defined as the number of PCR cycles required for the fluorescence signal to exceed the background detection threshold value. From this standard curve, it is possible to determine the log-linear range and efficiency. The efficiency is calculated using the following equation:

\[
E = 10^{\left(-\frac{1}{S}\right)}
\]  

(1)

where S = slope of the standard curve.
(iv) Quantification of RNA in clinical samples

RNA quantification was performed in duplicates for two sets of patient samples consisting of normal, tumour and liver metastases. The quantification is carried out using the same conditions and reaction mix as in the standard curve. In every run, a water control tube was also set up.

(v) Statistical analysis

One-way ANOVA was performed using SPSS for Windows Version 10.0. A P-value ≤ 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Table 1. A summary of statistical data describing the stability of gene expression levels in normal, tumour and metastatic colorectal cells in clinical samples; and tumour and metastatic cells in cell lines.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Origin of RNA (sample name)</th>
<th>F</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>Clinical samples (A1)</td>
<td>0.655</td>
<td>0.581</td>
</tr>
<tr>
<td></td>
<td>Clinical samples (p222)</td>
<td>0.771</td>
<td>0.537</td>
</tr>
<tr>
<td>GUS</td>
<td>Clinical samples (A1)</td>
<td>0.313</td>
<td>0.752</td>
</tr>
<tr>
<td></td>
<td>Clinical samples (p222)</td>
<td>0.856</td>
<td>0.508</td>
</tr>
<tr>
<td>GAPDH*</td>
<td>Cell lines (HCT116, M1, M2, M3, D1, M4)</td>
<td>5.974</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Clinical samples (p235)</td>
<td>389.308</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*GAPDH data was obtained from a previous study to show contrast of stability of expression between the housekeeping genes.

The results of the quantification of 18S and GUS were analyzed by one-way ANOVA and the P-values are displayed in Table 1. Both genes showed no difference in the mean of expression in normal, tumour and metastatic tissues as the P-values are all greater than 0.05. For comparison, GAPDH P-values are shown for tumour versus metastases (cell lines), and for normal, tumour and metastases (patient samples). The difference is statistically significant for cell lines and patient sample. Post-hoc tests were then carried out to determine which cells differed which others in particular. A Scheffé test reports GAPDH levels in M1 as significantly different from that in M3 (P < 0.05). For the patient samples, a Bonferroni test on mean GAPDH level shows tumour to be different from metastatic cells (P = 0.001), normal cells to be different from metastatic (P < 0.001) and normal to be different from tumour (P < 0.01).

From these results, GAPDH appears to regulated in colorectal carcinoma and their corresponding liver metastases. Not only is it regulated, it is strongly regulated in these cells. The strength of the difference can be inferred from the large F value of 5.94 in cell lines and 389.308 for p235 clinical samples. Therefore, GAPDH should not be used as a HKG for gene expression analysis in patient colorectal cancer tissues. GAPDH however, still has useful application in colorectal cell lines with the exception of M1 versus M3.

The statistical analysis for 18S and GUS reveals that both or either one of them can be used as an internal control for gene expression analysis in colorectal tissues. It appears that these two genes make good candidates for purposes of normalization under the conditions studied. However, there are drawbacks and limitations in using 18S rRNA. The strongest argument...
against it is that rRNA may not be representative of the cell mRNA. Studies have shown that rRNA varies independently in 7.5% of mammary adenocarcinomas. Furthermore, rRNA makes up about 70% of the cell RNA. Thus, small amounts of degradation in mRNA may not show up in rRNA copy number but would greatly affect the Ct readings of a rare transcript. This illustrates another shortcoming of using a HKG with a different abundance relative to the target gene. One way to solve this problem is to match a highly expressed target gene to a highly expressed HKG. In this study, three different HKGs with high (18S), moderate (GUS) and low (PBGD) were used, thus providing a choice of HKGs with a range of abundance level. However, consistency of PBGD transcript number has yet to be validated under these experimental conditions.

CONCLUSION

The results show that 18S and GUS are stably expressed across the cells used in the study and thus, can be used as accurate normalizers to detect a real change in gene transcript numbers when one exists, and to avoid concluding there is a change when there is actually none at all. This findings indicates that these genes can be used for future studies in the same cell types and experimental conditions but caution must be exercised when applying them to different cells or to the same cells under different treatment.

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REFERENCES