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Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes

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For interpretation of quantitative gene expression measurements in clinical tumor samples, a normalizer is necessary to correct expression data for differences in cellular input, RNA quality, and RT efficiency between samples. In many studies, a single housekeeping gene is used for normalization. However, no unequivocal single reference gene (with proven invariable expression between cells) has been identified yet. As the best alternative, the mean expression of multiple housekeeping genes can be used for normalization. In this study, no attempt was made to determine the gold-standard gene for normalization, but to identify the best single housekeeping gene that could accurately replace the measurement of multiple genes. Expression patterns of 13 frequently used housekeeping genes were determined in 80 normal and tumor samples from colorectal, breast, prostate, skin, and bladder tissues with real-time quantitative RT-PCR. These genes included, large ribosomal protein, β-actin, cyclophilin A, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerokinase 1, β-2-microglobin, β-glucuronidase, hypoxanthine ribosyltransferase (HPRT), TATA-box-binding protein, transferrin receptor, porphobilinogen deaminase, ATP synthase 6, and 18S ribosomal RNA. Principal component analysis was used to analyze these expression patterns, independent of the level of expression. Our approach identified HPRT as the single best reference gene that could be used as an accurate and economic alternative for the measurement of multiple housekeeping genes. We recommend this gene for future studies to standardize gene expression measurements in cancer research and tumor diagnostics until a definite gold standard has been determined.
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Quantitative RT-PCR is a frequently applied method to identify genes that correlate with tumor diagnosis or disease prognosis.[1,2] In this assay, it is mandatory to relate gene expression to the amount of tissue analyzed, that is, to normalize for optimal comparison of expression levels between tissue samples. Inaccurate normalization results in inadequate quantification and spurious conclusions.

Usually, cellular maintenance genes, the so-called housekeeping genes, are selected to normalize for the variability between clinical samples. These genes regulate basic and ubiquitous cellular functions and code, for example, for components of the cytoskeleton (β-actin), major histocompatibility complex (β-2-microglobulin), glycolytic pathway (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerokinase 1), metabolic salvage of nucleotides (hypoxanthine ribosyltransferase), protein folding (cyclophilin), or synthesis of ribosome subunits (rRNA). In many experiments, the expression of these genes is assumed invariable between cells of different samples and used as normalizer without proper validation. Other studies have shown, however, that their individual expression may vary as a result of neoplastic growth,[3,4] hypoxia,[5] or experimental treatment,[6–8] and may seriously influence correct interpretation of results.

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The 'gold-standard' normalization gene is still the subject of debate. Currently, the best option is to measure the expression of multiple housekeeping genes and normalize using their mean expression.

In cancer research, only few studies attempted to investigate the variation in expression of housekeeping genes between tissue samples. Mostly, only two or three candidate genes were compared.[3,4,9–11] In the present study, we attempted to identify a single housekeeping gene that could replace the measurement of multiple genes. Therefore, expression patterns of 13 ordinarily used housekeeping genes were investigated in 80 epithelial tissue samples (normal and tumor tissues). These samples represented colorectal, breast, prostate, skin, and bladder tissues, with tumors ranging from noninvasive to metastatic carcinomas. Quantitative RT-PCR was used for the generation of expression data. Principal component analysis (PCA), linear regression and difference-plot analysis were used to interpret data.

Materials and methods

Tissues

A total of 16 frozen tissues were selected for each organ (colon, breast, prostate, skin, bladder) from the tissue banks of the Departments of Pathology, Urology, Surgery, and Chemical Endocrinology (UMC Nijmegen, The Netherlands). For the colon, four normal tissues were selected together with 12 tumor tissues with increasing Dukes stage:[12] three Dukes A, three Dukes B, three Dukes C, and three Dukes D tumors. For the breast, two normal tissues were selected together with two fibroadenomas and 12 tumors with increasing pathological stage:[13] two T_{is} (carcinoma *in situ*), one stage 1, one stage 2A, two stage 2B, two stage 3A, two stage 3B, and two stage 4 tumors with metastases. For prostate, four normal tissues were selected together with three benign prostate hyperplasias, and nine tumor tissues with increasing TNM stage:[14] three T_2N_0, three T_3N_x, and three $T_4N_xM_x$-tumors. For the skin, three normal tissues, three normal nevi, three dysplastic nevi, three primary melanomas, and four melanoma metastases were selected. For the bladder, four normal urothelia were selected together with 12 urothelial cell carcinomas:[14,15] two pTa grade 1, two pTa grade 2, two pT1 grade 2, two pT2 grade 3, two pT3 grade 3, and two pT4 grade 3 with distant metastates. Total RNA was isolated from tissues and reverse transcribed to cDNA as described previously.[16]

Housekeeping Genes

A total of 13 housekeeping genes, used in many studies, were selected for gene expression analysis (Table 1). All genes are constitutively expressed in various tissues.[9,17,18] To our knowledge, all genes have independent functions in cellular maintenance, and regulation of their expression is assumed not to be related directly. Only GAPDH and PGK share an identical biochemical process in the cell, namely glycolysis.

Gene Expression Measurements

Measurement of the expression of all genes, except for PBGD and ATP6, was performed using the human endogenous control plate (Applied Biosystems). The human endogenous control plate is a 96-well PCR plate divided into 12 columns of eight identical wells. A total of 11 columns contain TaqMan primers and probes for the detection of 11 different housekeeping genes. The 12th column contains a fixed copy number of an artificial positive control sequence (APC) and corresponding primers and probe. If PCR inhibitors from the sample are present in the PCR reaction, the signal generated by the APC sequence will diminish. Samples containing inhibitors are excluded from further

Table 1 Selected housekeeping genes for gene expression analysis

Code	*Gene name*	*Abbreviation*	*Cellular function*
G1	Large ribosomal protein	LRP	Transcription
G2	β-actin	BACT	Cytoskeleton
G3	Cyclophilin A	CYC	Serine-threonine phosphatase inhibitor
G4	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Glycolysis enzyme
G5	Phosphoglycerokinase 1	PGK	Glycolysis enzyme
G6	β-2-microglobulin	B2M	Major histocompatibility complex
G7	β-glucuronidase	BGUS	Exoglycosidase in lysosomes
G8	Hypoxanthine ribosyltransferase	HPRT	Metabolic salvage of purines
G9	TATA-box-binding protein	TBP	Transcription by RNA polymerases
G10	Transferrin receptor	TfR	Cellular iron uptake
G11	Porphobilinogen deaminase	PBGD	Heme synthesis
G12	ATP synthase 6	ATP6	Oxydative phosphorylation
G13	18S ribosomal RNA	rRNA	Ribosome subunit

analysis. In the absence of inhibitors, the APC sequence should present a constant cycle threshold (C_t) value.

A measure of 10 μl of cDNA of each tissue sample ($n = 80$) was added to 315 μl H_2O and 325 μl Universal Master Mix (Applied Biosystems). This mixture was distributed over a row of 12 wells, with 50 μl mixture in each well. Consequently, 11 housekeeping genes and the APC sequence were amplified for each individual tissue sample with equal input of cDNA for each gene measurement. In total, 10×96-well PCR plates were used for all five tissue-types (5×16 samples). Increase of fluorescence was measured at every PCR cycle in each well by the ABI Prism 7700 sequence detection system (Applied Biosystems). Measurement of the expression of ATP6 and PBGD genes was described previously,[9,19] also using an equal input of cDNA. The expression of PBGD and ATP6 could not be measured in prostate tissues due to insufficient sample.

After PCR, the number of PCR cycles to reach the fluorescence threshold in each sample was defined as the cycle threshold (C_t). C_t values are proportional to the negative logarithm of the initial amount of input cDNA.[20] C_t values of 13 housekeeping genes in one tissue sample were directly related, since the input of cDNA was equal for each PCR reaction.

Statistical Analysis

Analysis for patterns of gene expression within the set of 13 housekeeping genes was performed with PCA using the SAS package (SAS Institute Inc.). PCA is a linear dimensionality reduction technique, which identifies (orthogonal) directions of maximum variance in the original data. The data are projected in a lower dimension, formed of (a subset of) components with the highest variance.[21,22] Principal components are therefore linear combinations of the original variables, orthogonal, and ordered with respect to their variance. The first principal component has the largest variance and represents better than any other linear combination the general differences/similarities between the housekeeping genes. In the analysis of the expression of different housekeeping genes, the housekeeping genes were used as the variables and the C_t values of the tissue samples as the observations. This will group housekeeping genes with similar variation in C_t (ie expression) patterns between all tissue samples. All PCA results were analyzed in two dimensions. To assess the reliability of the PCA analysis on the whole data set we used a split-sample technique to replicate the results. For this, the set of 80 tissues was divided into two subsets, comparable with respect to type of organ and degree of malignancy.

Results

Quality Control

In each endogenous control plate, C_t values for the APC sequence were constant with a within-run standard deviation of $C_t < 0.3$, demonstrating that PCR inhibitors were not present and that C_t values of all housekeeping genes could be compared. Only one out of 80 samples (a metastatic melanoma tissue sample) showed a significantly higher C_t-value (ie lower expression) from the others. In addition, one fibroadenoma of the breast contained a very low amount of RNA. Both samples were omitted from further analysis.

Principle of Expression-Profile Analysis

In our study, the cDNA generated from each sample was split into 13 equal parts for the measurement of 13 different housekeeping genes. The standardized cDNA input for the measurement of each gene linked expression patterns (represented by C_t values) in a sample directly. This provided the basis for our analyses. For example, when the expression patterns of two housekeeping genes were closely related, C_t values increased (or decreased) simultaneously between a series of tissue samples (depending on differences of cDNA amounts between the samples). Subsequently, housekeeping genes could be identified, which show similar expression patterns in all tissue samples. Closely related genes, which fluctuate concurrently, form a cluster with PCA.

Principal Component Analysis

The relationship between the expression patterns of 13 housekeeping genes was investigated mathematically with PCA of the C_t values of all 78 tissues. In the two-dimensional analysis, the first and second principal components explained 76 and 12% of the variability (88% in total). Analysis with more components was considered superfluous. Three different patterns of expression were observed (Figure 1). One pattern of expression was represented by ATP6 (G12), another by rRNA (G13), and the third pattern encompassed a cluster of 11 housekeeping genes (G1–G11). After repeating the PCA analysis on two halves of data sets (split-sample method), we observed the same clustering of genes in both data sets (data not shown).

Linear Regression and Difference Plot

The possibility that the expression of a single gene may represent the mean expression of the whole cluster of 11 genes was illustrated by plotting the expression (C_t value) of each individual housekeeping gene *vs* the mean expression (mean C_t) of

the remaining housekeeping genes for each sample. After linear regression, coefficients of correlation (r^2) were calculated, representing the relationship of the expression of each individual housekeeping gene with the mean expression of the other 10 genes. Coefficients of correlation were presented for each tissue-type separately and for all tissue-types together (Table 2). The power of the data for each tissue-type separately is lower (ie has a higher deviation) than the coefficient of correlation for all tissues together, since only 15 or 16 tissue samples were used for analysis per tissue-type. Clearly, hypoxanthine ribosyltransferase (HPRT) shows a very high correlation coefficient for each tissue-type separately ($r^2 \geq 0.90$), and the best score for all tissues together ($r^2 = 0.93$).

The absolute relationship between the expression of HPRT and the mean expression of the other genes can be quantified using a difference plot (Figure 2). Here, the difference between the C_t of HPRT and the mean C_t is plotted against the mean C_t. The mean difference ($C_t = 3.0$) represents the constant difference in level of expression between HPRT and the mean expression of the other genes: HPRT has an overall lower expression than the mean expression (3.0 extra PCR cycles are needed to reach fluorescence threshold). Important, however, is the deviation around the mean expression. This represents the accuracy of the HPRT expression measurement compared to the mean expression. For HPRT, two times the standard deviation from the mean difference is 1.3 C_t, showing that measurement of HPRT expression maximally deviates ± 1.3 C_t ($=$PCR cycles) from the expression measurement of the other genes together (for 95% of the tissue samples). The mean difference in expression with corresponding accuracy ($2 \times$ s.d.) is presented for all genes in Table 3.

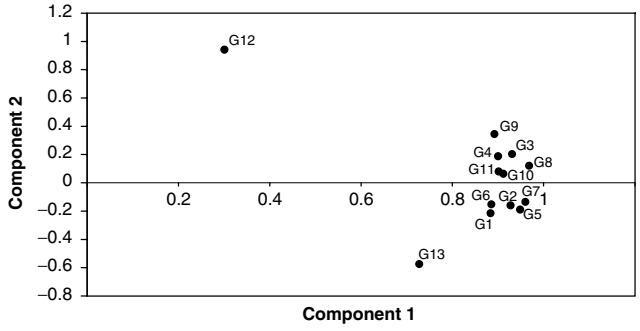

Figure 1 PCA of the expression data (C_t values) of 13 housekeeping genes in 78 tissue samples. The distance between genes (G1–G13) is expressed in relative units and inversely related to similarities in expression pattern.

Discussion

A major difficulty in studying differential gene expression is how to normalize for heterogeneity between tissue samples. Ideally, the gene transcript number is corrected for the number of cells analyzed. Unfortunately, counting of cells is not practical for solid epithelial tissues. Alternatively, total RNA can be quantified and used for normalization.[5,7,9,10,23,24] However, cellular RNA content may increase with increasing tumor aneuploidy.[25,26] Moreover, the majority of total RNA consists of ribosomal and mitochondrial RNA, hence it is not surprising that the expression of rRNA and ATP6 (a mitochondrial-coded gene) correlates with total

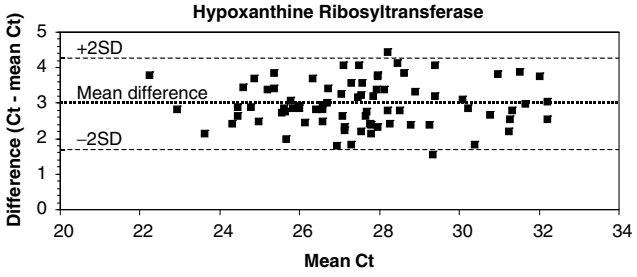

Figure 2 Difference plot, representing the accuracy of the expression of HPRT compared to the mean expression of 10 other housekeeping genes in all tissue samples.

Table 2 Coefficients of correlation between the expression of each individual gene and the mean expression of the remaining 10 genes

Gene code	*Abbreviation*	*Colon*	*Breast*	*Prostate*	*Skin*	*Bladder*	*All tissues*
G1	LRP	0.96	0.55	0.44	0.70	0.62	0.74
G2	BACT	0.92	0.86	0.86	0.94	0.84	0.82
G3	CYC	0.81	0.66	0.90	0.95	0.92	0.85
G4	GAPDH	0.74	0.82	0.80	0.79	0.90	0.76
G5	PGK	0.91	0.65	0.95	0.96	0.85	0.88
G6	B2M	0.62	0.53	0.62	0.70	0.71	0.76
G7	BGUS	0.74	0.83	0.96	0.97	0.84	0.91
G8	HPRT	0.92	0.90	0.92	0.95	0.95	0.93
G9	TBP	0.93	0.72	0.83	0.92	0.75	0.75
G10	TfR	0.89	0.88	0.97	0.73	0.81	0.81
G11	PBGD	0.87	0.93	NA	0.93	0.61	0.81

Data are represented for tissue-types separately and for all tissues together.
NA = not analysed.

Table 3 Independent relationship between the expression of each single housekeeping gene compared to the mean expression of the other 10 genes

Gene code	*Abbreviation*	*Mean difference* (C_t)	*Accuracy* ($2 \times s.d.$)
G1	LRP	−2.6	2.8
G2	BACT	−5.5	1.9
G3	CYC	2.3	2.0
G4	GAPDH	−4.2	2.3
G5	PGK	−0.3	1.9
G6	B2M	−2.2	2.5
G7	BGUS	1.7	1.4
G8	HPRT	3.0	1.3
G9	TBP	5.4	2.3
G10	TfR	2.0	2.0
G11	PBGD	0.6	1.7

The mean difference (C_t–mean C_t) represents the difference in height of expression, whereas the accuracy represents $2 \times$ the standard deviation.

RNA amount in some studies.[3,9] This does not prove that these genes are invariably expressed during tumorigenesis. In addition, total RNA quantification does not correct for RNA quality differences (eg due to storage), nor for differences in reverse transcriptase efficiencies between samples.[27] It is now generally accepted that gene expression levels should be normalized to an invariably expressed internal control gene that reflects differences in cellular input, RNA quality, and RT efficiency. However, how can such a gene be found when no gold standard is available to refer its expression to?

To circumvent this circular problem we used a different approach. We hypothesized that the mean expression of a large set of housekeeping genes with independent cellular functions would accurately reflect optimal normalization. Therefore, we chose 13 housekeeping genes with independent functions in cellular maintenance. This independence is important, because selection of genes that share identical biochemical pathways could bias analysis. A large set of epithelial tissue samples was selected ($n = 80$), representing five different organs, each spanning a range from normal tissues to metastatic carcinomas. Expression of each housekeeping gene was measured in the tissue samples with real-time quantitative PCR. No attempt was made to determine the gold-standard gene, but to select a single gene that could replace multiple gene measurements. PCA showed that the expression pattern of ATP6 and rRNA significantly differed from a large cluster of 11 genes and from each other. The difference in expression patterns between the three gene clusters may be associated with the different RNA polymerases that regulate their transcription: RNA polymerase I transcribes rRNA, mitochondrial RNA polymerase transcribes ATP6, and RNA polymerase II transcribes the other 11 genes. Since (1) PCA was not influenced by differences in expression levels between genes but recognized patterns only and (2) regulation of expression of the 13 housekeeping genes was (assumed to be) unrelated, we concluded that ATP6 and rRNA were poor normalization genes and both were excluded from further analysis.

Within the large cluster of the 11 remaining genes, the expression pattern of HPRT most accurately reflected the mean expression pattern of the other 10 genes. In the difference plot, an accuracy of ± 1.3 PCR cycles (C_t) implies that normalization may differ approximately two-fold using the expression of HPRT instead of the mean expression of all genes. This demonstrates that the expression of HPRT could be used as a simple and economic alternative for the measurement of 11 genes simultaneously. Although the combination of more than one gene may improve accuracy, for most research applications this will not be necessary. In exceptional situations, when only little RNA of a clinical sample is available (eg after microdissection) and the gene of interest has a high expression, HPRT expression may be measured (close to) negative and accurate normalization is not possible. In this situation, a high-expression housekeeping gene can be selected from Table 3. For instance, β-actin has a mean expression that is 360-fold higher than HPRT (8.5 PCR cycles difference to reach fluorescence threshold) and shows a good coefficient of correlation for colorectal and skin tissues (Table 2). Alternatively, when the gene of interest has intermediate or low expression, or in all situations when sufficient cells are present, a low-copy housekeeping gene controls best for RNA isolation efficiency, RNA quality, and RT-efficiency. HPRT then remains a good choice, since its expression is relatively low.

In a recent study by Vandesompele *et al*,[28] a similar method was presented using the geometric mean of the expression of 10 housekeeping genes. The design of their method was not suitable to identify a single normalization gene, but rather combinations of at least two genes that best represented the geometric mean. Even though most of their samples were derived from cell lines (neuroblastoma) and cell cultures (fibroblasts), hence very different in nature from our epithelial tissue samples, HPRT was frequently present in the best combination of genes. In addition, β-2-microglobulin was one of the worst scoring genes in their samples. This also corroborated our data.

Our study clearly demonstrates that the expression of the HPRT gene accurately reflects the mean expression of multiple commonly used housekeeping genes. For differential expression studies in cancer research, HPRT will be the most economic and accurate choice as single normalization gene.

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