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Validation of endogenous controls for gene expression analysis in microdissected human renal biopsies

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Validation of endogenous controls for gene expression analysis in microdissected human renal biopsies.

Background. The appropriate choice of an internal reference is critical for quantitative RNA analysis. However, no comparison of frequently used "housekeeping" genes is available for renal biopsy studies.

Methods. Microdissected biopsies from 165 patients, including a wide range of histopathologic diagnoses, were analyzed [immunoglobulin A (IgA) nephritis, membranous glomerulopathy, rapid progressive glomerulonephritis, minimal change disease, focal segmental glomerulosclerosis (FSGS), nephrosclerosis, diabetic nephropathy, interstitial nephritis, and controls]. Expression of three frequently used housekeeping genes [glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *18S rRNA*, and *cyclophilin A*] was examined by real-time reverse transcription-polymerase chain reaction (RT-PCR).

Results. Absolute expression values of reference genes obtained from the renal biopsies were related to each other. In tubulointerstitial compartment, a positive correlation coefficient (r) of 0.96 was observed between *18S rRNA* and *cyclophilin A*. However, a subset of samples showed lower expression levels for *GAPDH* in relation to *18S rRNA* or c*yclophilin A*, resulting in a decrease to $r = 0.77$ and $r = 0.73$, respectively, consistent with considerable mRNA regulation of *GAPDH*. In glomerular samples, a comparable low correlation between *GAPDH* versus *18S rRNA* ($r = 0.75$) was obtained.

Conclusion. Using a single housekeeper gene as reference for renal biopsy studies, differences in gene expression ratios may reflect regulation of the internal control rather than the mRNA under investigation. Relating the gene expression to several housekeepers in parallel (i.e., *18S rRNA* and *cyclophilin A*) should result in robust expression data.

With the human genome in common domain and the development of highly effective gene expression quantification technologies, mRNA analysis of disease processes has become widely available [1]. Quantitative "real-time" detection methods using fluorogenic oligonucleotides like the TaqMan® reverse transcription-polymerase chain reaction (RT-PCR) technique allow for the rapid and accurate quantification of mRNA templates even in minimal tissue samples (e.g., renal biopsies).

For quantitative mRNA studies, choosing a valid internal control for monitoring intersample variation is mandatory. Genes, which are coexpressed with the target gene, but are not transcriptionally regulated by the specific experimental design, are used as endogenous controls and are referred to as "housekeeping genes."

Published gene expression studies of human kidney biopsies have predominantly used glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or *β-actin* as internal standards for RT-PCR. These genes are expressed at relatively high levels and have served as reference genes in Northern blot analysis for the past two decades. Despite the common use of these control templates, no comprehensive analysis has been performed to confirm their stable expression in renal disease. In addition, there is recent evidence of potential regulation of *GAPDH* as well as *β-actin* [2].

To validate internal controls for the analysis of gene expression in human nephropathies, we compared the expression of three commonly used housekeeping genes, *GAPDH*, *18S rRNA*, and *cyclophilin A*, in tubulointerstitial as well as glomerular compartments of human renal biopsies.

METHODS

Human biopsies

Human kidney biopsies were obtained via the framework of the European Renal cDNA Bank (ERCB) (see **Appendix** for listing of members) from patients after informed consent and with approval of local ethical committees. To standardize storage conditions, a commercially available RNase inhibitor (RNAlater, Ambion, Austin, TX, USA) was used as previously described [3]. Biopsies from 165 patients, including a wide range of

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histopathologic diagnoses were analyzed in this study: immunoglobulin A (IgA) nephritis (N = 35), membranous glomerulopathy (N = 28), rapid progressive glomerulonephritis (N = 20), benign nephrosclerosis (N = 16), minimal-change disease (N = 14), diabetic nephropathy (N = 13), focal segmental glomerulosclerosis (FSGS) (N = 9), interstitial nephritis (N = 8), and controls (N = 22). Controls were divided into following subgroups: unaffected regions from tumor nephrectomies (N = 14) and pretransplantation kidney biopsies during cold ischemia time from cadaveric (N = 4) as well as living donors (N = 4).

Microdissection

Microdissection of renal biopsies stored in RNAlater was performed manually under a stereomicroscope using two dissection needle holders. This method offers a reliable and fast dissection of nephron segments into glomeruli and tubulointerstitial fragments (for details see [3]).

RNA isolation and RT

A silica gel-based RNA isolation protocol (RNeasy-Mini; Qiagen, Hilden, Germany) was followed by RT in a 45 μL volume, containing 9 μL buffer, 2 μL 0.1 mol/L dithiothreitol (DTT) both from Gibco BRL Life Technologies (Gaithersburg, MD, USA), 0.9 μL 25 mmol/L desoxynucleoside triphosphate (dNTP) (Amersham Pharmacia, Freiburg, Germany), 1 μL RNase inhibitor (RNasin; Promega, Mannheim, Germany), 0.5 μL Microcarrier (Molecular Research Center, Cincinnati, OH, USA), 1 μg random hexamers (2 mg/mL stock; Roche, Mannheim, Germany) and 200 U reverse transcriptase (Superscript I; Invitrogen Life Technologies, Karlsruhe, Germany) for one hour at 42°C.

Real-time quantitative RT-PCR

Real-time RT-PCR was performed on a TaqMan® ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany) using heat-activated TaqDNA polymerase (Amplitaq Gold; Applied Biosystems). Cycle conditions were as follows: after an initial hold of 2 minutes at 50°C and 10 minutes at 95°C, the samples were cycled 40 times at 95°C for 15 seconds and at 60°C for 60 seconds. For all quantitative analyses, the cDNA content of each sample was compared with another sample following the ΔΔ median threshold cycle number (C_t) technique [4]. Similar amplification efficiencies for all templates were demonstrated by analyzing serial cDNA dilutions showing a slope value of log input cDNA amount versus (C_t target A − C_t target B) of <0.1.

All assays were performed in parallel after RT, no duplex assays were used.

Commercially available predeveloped TaqMan® reagents with optimized primer and probe concentrations for human *GAPDH*, *18 S rRNA*, and *cyclophilin A* were obtained from Applied Biosystems. The primers and probes for *GAPDH* and *cyclophilin A* are cDNA-specific, whereas the assay for *18S rRNA* may detect contaminating genomic DNA. Comparing reverse transcriptase–negative with transcriptase-positive samples, a ΔC_t of ≥10 was observed corresponding to a negligible contamination of genomic DNA below 0.1%, as previously demonstrated for the above RNA preparation on microdissected renal specimen [3]. All PCR reactions were performed in duplicate. Controls consisting of bidistilled H_2O were negative in all runs.

Statistical analysis

Data are given as absolute values or mean ± SD. All data were analyzed using the SPSS software (version 10.0, SPSS, Inc., Chicago, IL, USA). Mean differences of nonparametric data were analyzed by the Mann-Whitney U test. Kruskal-Wallis test was used to compare means among more than two groups. Correlations, including correlation coefficients and confidence intervals, were assessed by linear regression analysis. P less than 0.05 was considered to be statistically significant.

RESULTS

Validation of endogenous controls in microdissected tubulointerstitial compartments

Different expression levels were demonstrated for the evaluated housekeeping genes in microdissected tubulointerstitial compartments. The highest expression level was seen for *18S rRNA* (median C_t = 13.89 ± 2.06 SD), followed by *GAPDH* (median C_t = 22.57 ± 2.61) and *cyclophilin A* (median C_t = 30.96 ± 2.20). Twelve samples (7.27%) were outside the 95% CI of the *GAPDH/18S rRNA*, as well as the *GAPDH/cyclophilin A*, mRNA ratio (Fig. 1) [(r = 0.77; P < 0.01) for *GAPDH/18S rRNA* and (r = 0.73; P < 0.01) and for *GAPDH/cyclophilin A*].

Absolute mean mRNA expression levels ($\log_{10}$-transformed) are demonstrated for this subset of biopsies compared to expression levels of the remainder of biopsies in Figure 2.

The mean value of *GAPDH* expression is significantly lower in the 12 differentially regulated samples compared to the remaining biopsies, indicating a significant *GAPDH* mRNA down-regulation in this subset of probes. For *18S rRNA* and *cyclophilin A* gene expression levels did not change between the groups.

Further investigation of the subset of biopsies with *GAPDH* regulation did not reveal an obvious common histopathologic nor clinical pattern [IgA nephritis (N = 3), membranous glomerulopathy (N = 3), diabetic nephropathy (N = 2), rapid progressive glomerulonephritis (N = 2), unaffected regions from tumor nephrectomies (N = 1), and pretransplantation kidney biopsies from cadaveric donors (N = 1)]. In addition, no systematic

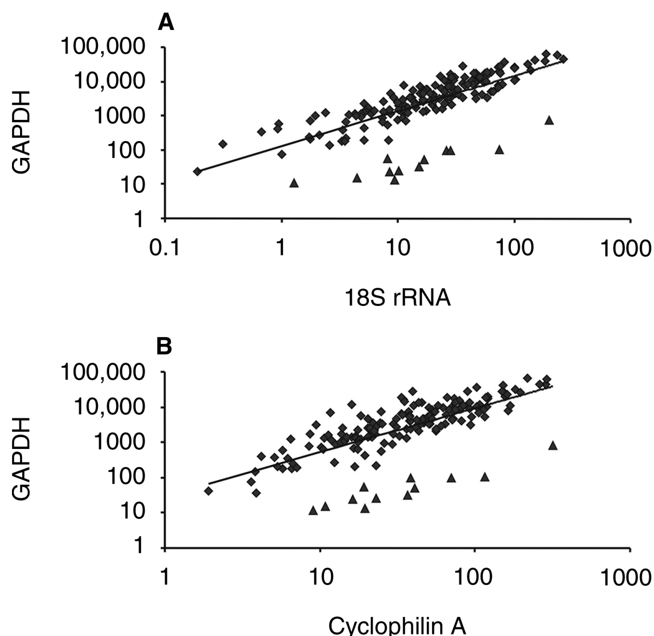

Fig. 1. Expression of housekeeping genes in microdissected tubulointerstitial compartment. (*A*) Correlation of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) vs. *18S rRNA*. (*B*) Correlation *GAPDH* vs. *cyclophilin A*. For correlation of *GAPDH* (y-axis, log_{10}-transformed) vs. *18S rRNA* (x-axis, log_{10}-transformed) mRNA expression values (r = 0.77, $P < 0.01$, $R^2_{corr} = 0.589$) were determined (A). Comparable results were obtained from a linear regression analysis of *GAPDH* (y-axis, log_{10}-transformed) vs. *cyclophilin A* (x-axis, log_{10}-transformed) mRNA expression values ($r = 0.73$, $P < 0.01$, $R^2_{corr.} = 0.526$) (B). In both correlation plots, 12 samples [7.27% of 165 examined biopsies (▲)] were outside the 95% CI.

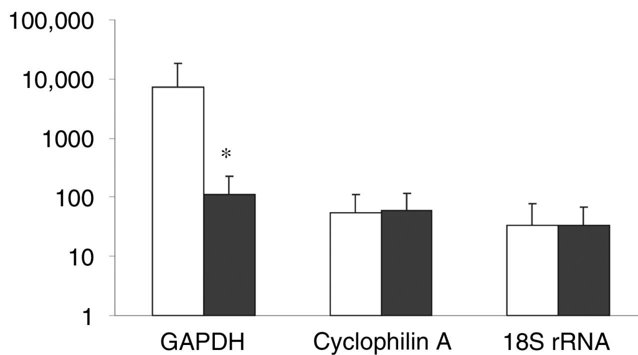

Fig. 2. Absolute mean mRNA expression levels (log_{10}-transformed) for three tested internal standards. Absolute mean mRNA expression levels (log_{10}-transformed) for a subset of biopsies compared to corresponding expression levels of the rest of biopsies: The mean value of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression is significantly lower in the 12 differentially regulated samples (■) compared to the remaining samples (□), indicating a *GAPDH* mRNA repression in this subset of biopsies. For *18S rRNA* and *cyclophilin A* gene expression levels did not change between the two groups. *$P < 0.02$.

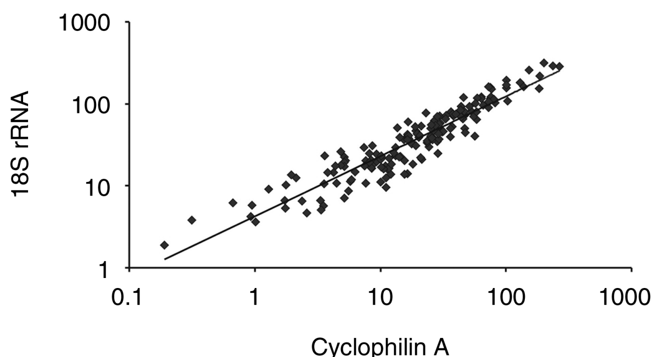

Fig. 3. Expression of housekeeping genes in microdissected tubulointerstitial compartment. Correlation of *18S rRNA* vs. *cyclophilin A*. Linear regression analysis of *18S rRNA* (y-axis, log_{10}-transformed) vs. *cyclophilin A* (x-axis, log_{10}-transformed) mRNA expression values revealed a stringent correlation for all analyzed biopsies ($r = 0.962$, $P < 0.01$, $R^2_{corr} = 0.925$).

technical differences could be identified in this subset of biopsies (i.e., no difference in RNA quality or quantity, duplicate variability or center bias).

A stringent correlation of *18S rRNA*/*cyclophilin A* was obtained in the tubulointerstitial compartment for all analyzed biopsies ($r = 0.96$, $P < 0.01$) (Fig. 3).

Validation of endogenous controls in microdissected glomeruli

Analyses of microdissected glomeruli showed median $C_t = 19.36 \pm 2.31$ for *18S rRNA*, $C_t = 28.96 \pm 2.38$ for *GAPDH*, and $C_t = 34.67 \pm 1.54$ for *cyclophilin A*. This reflects lower RNA content of the glomerular samples compared to the tubulointerstitial specimen. As the amount of microdissected glomeruli was limited in our assay [3], amplification efficiency (i.e., for *cyclophilin A*, it could not be increased even with optimized primer and probe concentrations). As a consequence of the low abundance in the available glomerular compartment, *cyclophilin A* could not be used as an endogenous control in microdissected glomeruli. Furthermore, when choosing an internal reference as housekeeper gene, ΔC_t should be as small as possible. The correlation of *GAPDH* versus *18S rRNA* mRNA expression yielded comparable results to the tubulointerstitial compartment ($r = 0.75$, $P < 0.01$).

DISCUSSION

Real-time RT-PCR is a highly sensitive method for mRNA quantification with a wide linear range. This approach allows for the comparison of high- and low-abundant genes in one assay without the need for adjusting starting material quantities. It is currently the preferred method for gene expression quantification (e.g., for a limited number of target genes and for confirmation of cDNA array expression studies).

The so-called housekeeping genes serve as internal controls for quantification (i.e., as a reference for the target transcript). For the identification of internal reference genes, a comparison of expression levels for suitable housekeeping genes from microarray experiments seems to be the gold standard [5]. As more and more array studies evaluating gene expression profiles in renal dis-

Table 1. Internal controls used for reverse transcription-polymerase chain reaction (RT-PCR) gene expression analysis of renal tissue

Internal control "housekeeping gene"	Publications using this housekeeper
GAPDH	66 (66%)
β-actin	19 (19%)
18S rRNA	4 (4%)
Cyclophilin A	2 (2%)
Other	9 (9%)

GADPH is glyceraldehyde-3-phosphatase dehydrogenase.
[a] Housekeeping genes employed in RT-PCR gene expression analyses of kidney biopsies were determined in a PubMed National Library of Medicine search for the years 1999 to 2002 with the following key words: kidney, RT-PCR, gene expression

eases are published, these data sets can be used to identify adequate internal controls for the respective material. Microarray experiments are powerful research tools but have their own pitfalls. They are expensive, require a certain infrastructure, and many investigators are not experienced in the complex analysis and interpretation of the multitude of data obtained. Furthermore, selected gene expression patterns have to be confirmed by RT-PCR. Therefore, DNA array and real-time RT-PCR technology can be considered as complimentary approaches and array systems will not replace the more economic and versatile quantitative real time techniques in the near future.

For RT-PCR experiments, various housekeeping genes have been used to quantify gene expression in different samples. Gene expression studies of human kidney biopsies published in the years 1999 to 2002 predominantly used *GAPDH* as an internal standard for RT-PCR studies (see Table 1).

However, several reports indicate that expression of housekeeping genes can vary across tissues and cell types during cell proliferation and organ development [2]. To address the problem of housekeeper regulation, a systematic study of the expression of internal standards in renal biopsies is required. Therefore, *GAPDH*, *18S rRNA*, and *cyclophilin A* mRNA levels were determined in a large sample of microdissected renal biopsies. *β-actin* was not included in the study as regulation of this gene has already been demonstrated [6].

Microdissected tubulointerstitial specimen of kidney biopsies showed considerable variation in the expression levels of *GAPDH*, *18S rRNA*, and *cyclophilin A*. In a subset of biopsies, repression of *GAPDH* compared to *18S rRNA* and *cyclophilin A* was demonstrated.

These RT-PCR data are consistent with cDNA array expression profiles of total human kidneys, showing a remarkable variation in *GAPDH* mRNA expression (own unpublished observations). The differential regulation of *GAPDH* is in concordance with the function of this protein, including relevance for endocytosis, translational control, DNA replication and repair, apoptosis, and glycolysis [7]. *GAPDH* mRNA is elevated in various cancer tissues, including renal cell carcinoma [8, 9], correlating with the aggressiveness of the tumor [10]. *GAPDH* mRNA levels are altered in cultured cells in response to various stimuli (i.e., hypoxia, insulin, dexamethasone, mitogens, and epidermal growth factor) [11].

rRNAs are frequently used as internal controls for quantification experiments, particularly since stable expression levels of *18S rRNA* and *28S rRNA* relative to other housekeeping genes have been described for rat, mouse, and human tissues [12–14]. However, *18S* and *28S rRNA* are distinct from messenger RNAs, constituting up to 80% of total cellular RNA. rRNAs are transcribed by a specific RNA polymerase and their transcription can be affected by biologic factors, like cholera toxin or the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate, and drugs [15]. Furthermore, rRNAs can only serve as housekeepers for total RNA preparations, whereas mRNA enrichment protocols deplete rRNA in an unpredictable manner.

Cyclophilin A, a member of a highly conserved, ubiquitous protein family and originally isolated as the main cyclosporin A–binding protein [16] was analyzed as the third endogenous control. Its expression is not restricted to a specific cell type [17] and might therefore be useful as a housekeeper for comparative quantification of mRNA by RT-PCR [18]. However, since multiple cyclophilin-homologous sequences representing related genes or pseudogenes with high-sequence homology are present in mammalian genomes [19, 20], this housekeeper should be primarily used in RNA preparations with negligibly low genomic DNA contamination [2]. A further limitation of *cyclophilin A* as housekeeping gene for RT-PCR analysis of the glomerular compartment is the low abundance of its mRNA compared to *GAPDH* and *rRNA*.

CONCLUSION

All reference genes have the potential of differential regulation under certain conditions. Comparing *GAPDH*, *18S rRNA*, and *cyclophilin A* expression in a large cohort of microdissected kidney biopsies, *18S rRNA* and *cyclophilin A* can be recommended for the use of gene expression analysis of the tubulointerstitial compartment and most likely for whole, nonmicrodissected kidney biopsies. Moreover, the parallel determination of two or more housekeeping genes in a given sample population or experimental condition is crucial to allow for the detection of any systematic bias. This is of particular relevance, as accumulating expression data on human biopsies offer a unique resource for the establishment of molecular diagnostic markers and may facilitate the identification of novel pathogenic pathways in renal diseases [21].

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APPENDIX

Members of the European Renal cDNA Bank (ERCB)

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