

Robust MicroRNA Stability in Degraded RNA Preparations from Human Tissue and Cell Samples

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BACKGROUND: RNA integrity is the essential factor that determines the accuracy of mRNA transcript measurements obtained with quantitative real-time reverse-transcription PCR (RT-qPCR), but evidence is clearly lacking on whether this conclusion also applies to microRNAs (miRNAs). We evaluated this issue by comparative analysis of the dependence of miRNA and mRNA measurements on RNA integrity in renal and prostate samples, under both model and clinical conditions.

METHODS: Samples of total RNA isolated from human renal tissue and Caki-2 cells, as well as from prostate tissue and LNCaP cells, were incubated at 80 °C for 5–240 min. We subsequently determined the RNA integrity number (RIN) and used RT-qPCR to measure various miRNAs (miR-141, miR-155, miR-200c, and miR-210 in renal samples, and miR-96, miR-130b, miR-149, miR-205, and miR-222 in prostate samples). We similarly measured mRNAs encoded by *CDH16* (cadherin 16, KSP-cadherin), *PPIA* [peptidylprolyl isomerase A (cycophillin A)], and *TBP* (TATA box binding protein) in renal samples, and *HIF1A* [hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)], *HPRT1* (hypoxanthine phosphoribosyltransferase 1), and *KLK3* (kallikrein-related peptidase 3; also known as *PSA*) in prostate samples. Additionally, we quantified selected miRNAs and mRNAs in samples of RNAs with different RIN values that we isolated from clinical samples. The effect of RIN on the miRNA and mRNA data was assessed by linear regression analysis and group comparison.

RESULTS: The heat-incubation experiments of cell line and tissue RNAs showed that RIN values had negligible or no effect on miRNA results, whereas all mRNAs gradually decreased with decreasing RIN values. These

findings were corroborated by our findings with clinical samples.

CONCLUSIONS: Our results suggest the stability of miRNAs to be generally robust, which makes feasible accurate miRNA measurements with RT-qPCR, even in degraded RNA preparations for which reliable mRNA analyses are commonly inapplicable.

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MicroRNAs (miRNAs)⁵, small nonprotein-coding RNA molecules of 20–22 nucleotides, have important regulatory functions in various basic biological processes (1, 2). One miRNA can target about 200 mRNAs, whereas a single mRNA can be controlled by several miRNAs (3). About 30% of human mRNAs are probably regulated by miRNAs (4). To date, 721 human miRNAs have been identified (<http://www.mirbase.org>; release 14, September 2009), and computer simulations have predicted the existence of approximately 1000 miRNAs (5).

The contribution of miRNAs in various diseases, especially cancer, due to their interplay with target genes in cell proliferation, apoptosis, and differentiation has recently been demonstrated in numerous expression-profiling studies and functional experiments (1, 2, 6–12). Several mechanisms, including genetic and epigenetic alterations, can lead to either increased or decreased miRNA production (13). Downregulated miRNAs could have organ- and tissue-dependent oncogenic or tumor-suppressive properties (14). Thus, altered miRNA production profiles and the identification of their targets in cancer imply the high potential of miRNAs, not only as diagnostic, predictive, and prognostic markers, but also as a starting point in the development of new therapeutic strategies (15).

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Received December 3, 2009; accepted March 19, 2010.

Previously published online at DOI: 10.1373/clinchem.2009.141580

⁵ Nonstandard abbreviations: miRNA, microRNA; RT-qPCR, quantitative real-time reverse-transcription PCR; RIN, RNA integrity number; ccRCC, clear cell renal cell carcinoma; PCa, prostate cancer; miR-210, miRNA 210; MIQE, minimum information for publication of quantitative real-time PCR experiments; Cq, quantification cycle; FFPE, formalin-fixed, paraffin-embedded.

In this regard, reliable analyses of miRNA production are the prerequisite for further research progress. Several methods, such as *in situ* hybridization, ribonuclease protection assays, northern blotting, bead-based flow cytometry, microarrays, and quantitative real-time reverse-transcription PCR (RT-qPCR), have been developed to analyze miRNA production (16), with the last 2 approaches being the ones primarily used. In contrast to the microarray platform, RT-qPCR used with so-called stem-loop primers is a quantitative method for measuring miRNA and provides high specificity and sensitivity (17).

Experiments with RT-qPCR have shown that RNA degradation, which leads to mRNA cleavage, profoundly affects the accuracy of quantifying mRNA transcripts (18–21). The processes of tissue sampling in the clinic and of RNA isolation and handling in the laboratory are the critical determinants for obtaining intact RNA (22). The extent of RNA degradation can be estimated with the so-called RNA integrity number (RIN) (23), which characterizes RNA integrity on a continuous scale from 1 (RNA completely degraded) to 10 (RNA without degradation). The RIN evaluates not only the ratio of 28S to 18S ribosomal RNA but also other critical regions of the RNA electropherogram. This or an equivalent approach for evaluating RNA integrity was recently recommended as the standard operational procedure for assessing RNA quality, thereby facilitating comparisons of downstream experiments (24, 25). Although the RIN is not generally applicable and is both tissue and organ dependent, values >5 in mammalian RNA samples have been suggested as a minimum for reliable mRNA measurements (21). On the other hand, to the best of our knowledge no systematic studies exist on whether this preanalytical issue also applies to miRNA measurements. Moreover, one can assume that the short lengths of miRNAs make them less susceptible to degradation than the longer mRNAs. Concrete data have not been available, however.

Therefore, the aim of this study was to investigate (a) how RNA integrity influences miRNA measurements by RT-qPCR and (b) whether meaningful quantitative miRNA data can be obtained from degraded RNA samples. For these purposes, we measured miRNAs both in model experiments in which we artificially degraded RNA samples isolated from renal and prostate cells and tissues, and in corresponding clinical samples with different RIN values. We then compared the results with mRNA data. On the basis of our previous quantitative studies of miRNAs in clear cell renal cell carcinoma (ccRCC) and prostate cancer (PCa) (26, 27), we studied both up-regulated miRNAs (miR-210 and miR-155 for ccRCC; miR-96 and miR-130b for PCa) and down-regulated miRNAs (miR-141 and

miR-200c for ccRCC; miR-149, miR-205, and miR-222 for PCa) characteristic of the 2 cancer entities.

Materials and Methods

CELL LINES AND TISSUE SAMPLES

The human ccRCC cell line Caki-2 (HTB-47, obtained from the ATCC) and the human PCa cell line LNCaP (ACC 256, obtained from the German Collection of Microorganisms and Cell Cultures). Both cell lines were cultured under the manufacturer's standard conditions and harvested at 80%–90% confluence.

Tissue samples were obtained from previously untreated ccRCC and PCa patients at the time of radical nephrectomy and radical prostatectomy, respectively. The ethics committee of the hospital approved the use of patient samples. Tumor stage and grade were established according to the 2002 TNM classification system but were not of primary interest in this study. Immediately after surgery, tissue samples were snap-frozen in liquid nitrogen and stored at -80°C . Afterward, histologically verified and matched malignant and non-malignant samples from the same organ were prepared as described previously and transferred into RNAlater RNA Stabilization Reagent (Qiagen) (26, 27). Only areas with >90% tumor were collected as malignant samples. RNAlater-stabilized tissue samples were stored at -20°C until RNA isolation, which was performed within 2 months of tissue procurement.

ISOLATION OF TOTAL RNA

We used the miRNeasy Mini Kit (Qiagen) to isolate total RNA, including miRNAs. We disrupted several million cells or 20–50 mg of tissue with 700 μL QIAzol Lysis Reagent in a TissueLyser (Qiagen) at 30 Hz for 2×1 min. An on-column DNase digestion step was included and carried out according to the manufacturer's instructions. RNA was eluted from the spin-column membrane with the ultrapure water supplied in the kit. This ultrapure water was used for all subsequent dilutions of RNA samples. RNA yield and purity were evaluated with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies/Thermo Scientific) via absorbance measurements at 260 and 280 nm. A_{260}/A_{280} ratios between 1.96 and 2.04 were obtained for all samples of isolated RNA, confirming their high purity. RNA integrity was assessed with a 2100 Bioanalyzer (Agilent Technologies). Samples of isolated total RNA were stored at -80°C before use.

IN VITRO RNA-DEGRADATION EXPERIMENTS

We prepared 10 μL of equivalent concentrations (800 ng/ μL ; diluted in ultrapure water as described above) of the respective RNA pools from cell lines and tissue samples and incubated them for different times at

80 °C in a thermal block cycler with a heated lid (Bio-metra). We then prepared renal and prostate RNA pools, which contained a mixture of equal amounts of 800 ng/ μ L RNA from 10 matched malignant and non-malignant tissue samples. This procedure was chosen to measure both the up- and down-regulated miRNAs typical of the corresponding tissues (26, 27), as mentioned earlier, and to compensate for potential intraindividual variation in miRNA concentration as much as possible. RNA concentrations of the original solutions and dilutions, as well as the final concentrations of the mixture, were monitored by NanoDrop spectrophotometry so that the pools contained equal RNA amounts from every sample. The heat incubation was stopped by transferring the tubes into an ice bath; the tubes were then centrifuged at 4 °C and stored at –80 °C until analysis.

REAL-TIME RT-qPCR METHODOLOGY

The study was conducted according to MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (24). Details are provided in Table 1 of the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol56/issue6>.

cDNA SYNTHESIS OF miRNAs AND mRNAs

The TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and the Transcriptor First Strand cDNA Synthesis Kit (Roche) were used according to the manufacturer's instructions (see Supplemental Text 1 in the online Data Supplement).

QUANTIFICATION OF miRNAs AND mRNAs

The qPCR measurements were carried out on the LightCycler 480 Instrument (Roche) in white 96-well plates (Roche) with a reaction volume of 10 μ L and runs up to 45 cycles. The heating and cooling ramping rates were set automatically by the instrument. TaqMan MicroRNA assays (Applied Biosystems) were used to measure miRNAs (see Supplemental Text 1 and Table 2C in the online Data Supplement). Details on the measurement of mRNAs encoded by *CDH16*⁶ (cadherin 16, KSP-cadherin), *HIF1A* [hypoxia-inducible factor 1, α subunit (basic helix-loop-helix transcription factor)], *HPRT1* (hypoxanthine phosphoribosyltransferase 1), *PPIA* [peptidylprolyl isomerase A (cyclophilin A)], *KLK3* (kallikrein-related pepti-

dase 3; also known as *PSA*), and *TBP* (TATA box binding protein) are provided in Supplemental Text 1, Table 2, A and B, and Fig. 1, A and B, of the online Data Supplement. A nontemplate control, a cDNA mixture as secondary calibrator, and another one as a run control were conducted on each plate as control assays. All cDNA samples were measured in triplicate, and mean values for the quantification cycle (*C_q*) were used for calculations. To quantify mRNAs in the prostate and kidney samples as well as miRNAs in the kidney samples, we generated calibration curves from cDNA dilutions. We also used amplicon dilutions to generate calibration curves in order to obtain a wide dynamic range of miRNA measurements of the prostate samples (see Table 3 in the online Data Supplement). The samples for the heat-degradation experiments were run on a single plate to minimize analytical variation. The repeatability of the measurements of all analytes was reflected in CVs of <8% (see Table 4 in the online Data Supplement).

DATA PRESENTATION AND STATISTICAL ANALYSIS

Gene expression in the RNA-degradation experiments was calculated as *C_q* values. Gene expression in the clinical samples from nonmalignant renal and prostate samples was calculated from the calibration curves and expressed in arbitrary concentration units. The use of equal amounts of total RNA in the particular experiments (as described above) allowed direct comparisons of the respective gene expression data in the experiments.

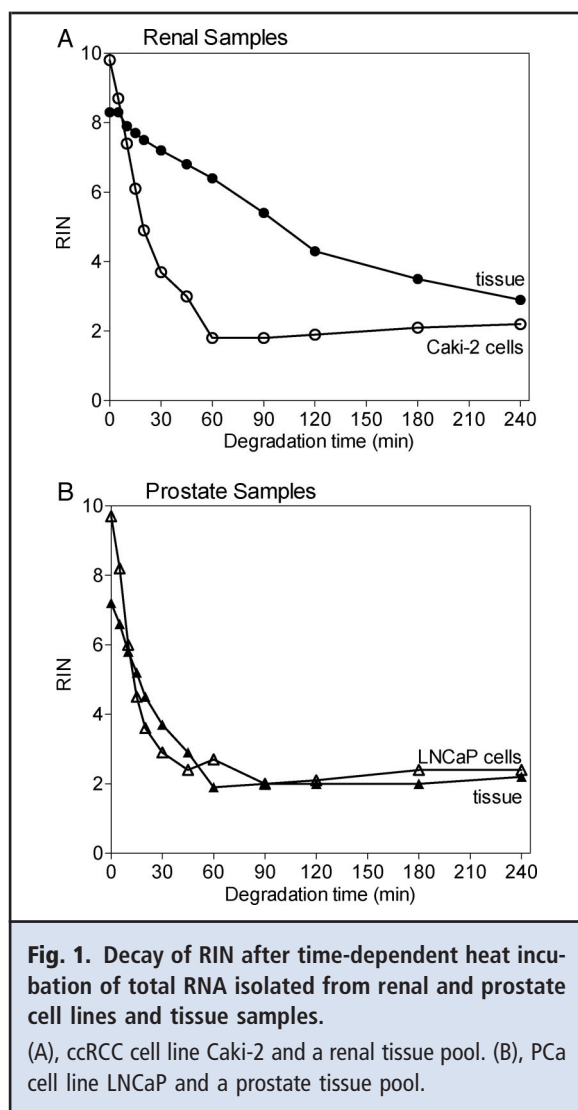
For statistical analyses, GraphPad Prism software (version 5.02; GraphPad Software) was used. Mann–Whitney *U*-tests and linear regression analyses were performed. The statistical significance of the slope was tested by its deviation from 0, with a *P* value <0.05 considered statistically significant. Sample size calculations and power analyses were carried out with GraphPad StatMate 2.0 (see Supplemental Text 2 in the online Data Supplement).

Results

IN VITRO HEAT-DEGRADATION KINETICS OF RNA ISOLATED FROM RENAL AND PROSTATE CELL LINES AND TISSUE SAMPLES

Fig. 2 in the online Data Supplement and Fig. 1 illustrate the degradation of RNA samples after incubation at 80 °C. The time-dependent decreases in RIN for RNA samples from Caki-2 and LNCaP cells, as well as from prostate tissue, were nearly equal (Fig. 1, A and B). The lowest measurable RIN value (approximately 2.0) was achieved after 60 min of heat incubation. By contrast, RNA from renal tissues was more resistant to heat degradation, with a RIN value of 2.9 after 240 min of heat treatment (Fig. 1A).

⁶ Human genes: *CDH16*, cadherin 16, KSP-cadherin; *HIF1A*, hypoxia-inducible factor 1, α subunit (basic helix-loop-helix transcription factor); *HPRT1*, hypoxanthine phosphoribosyltransferase 1; *PPIA*, peptidylprolyl isomerase A (cyclophilin A); *KLK3*, kallikrein-related peptidase 3; *TBP*, TATA box binding protein; *RN18S1*, RNA, 18S ribosomal 1; *MIR155HG*, MIR155 host gene (non-protein coding).



miRNA IN RNA SAMPLES DEGRADED IN VITRO

miRNA was measurable by RT-qPCR in heat-degraded RNA samples with RIN values up to approximately 3.0. We investigated 4 miRNAs characteristic of renal tissue (miR-141, miR-155, miR-200c, miR-210) (26) and 5 miRNAs characteristic of prostate tissue (miR-96, miR-130b, miR-149, miR-205, miR-222) (27) (Fig. 2). We calculated the linear regression equations of Cq vs RIN values and plotted the regression lines.

The concentrations of miRNAs measured in the degraded RNAs from Caki-2 cells, LNCaP cells, and prostate tissue samples, as well as miR-141 and miR-210 in renal tissue samples were stable, independent of the RIN value (Fig. 2). The slopes of the regression lines showed no significant deviation from 0 (see Table 5 in the online Data Supplement). Only the slopes of the regression lines for miR-155 and miR-210 in heat-

degraded RNA samples from renal tissue differed significantly from 0 (Fig. 2B; see Table 5 in the online Data Supplement). Calculated from the regression equations for these 2 miRNAs, the differences in Cq between untreated and degraded RNA samples with the lowest RIN values were 0.18 for miR-210 and 0.29 for miR-155. If one considers the concentrations of these 2 miRNAs as a percentage of that of the untreated RNA (100%; RIN = 8.3), all measured samples up to a RIN value of 2.9 showed a mean (SD) relative concentration of 104% (5.86%) for miR-210 and 102.2% (10.5%) for miR-155. This variation can be considered to be within the range of analytical precision.

mRNA IN RNA SAMPLES DEGRADED IN VITRO

To illustrate the dependence of mRNA concentration on RNA integrity in contrast to the independence of miRNA concentration, we measured the expression of tissue-specific genes in heat-degraded RNA samples. We selected *CDH16* and reference genes *PPIA* and *TBP* for renal samples and selected *KLK3*, *HIF1A*, and reference gene *HPRT1* for prostate samples (Fig. 3). Cq values increased with decreasing RIN values in all cases, and the slopes of the regression lines were significantly different from 0 for all genes (see Table 5 in the online Data Supplement). Differences between the various genes and differences between the RNA sources (from cell lines or tissue samples) were clearly evident. For example, expression of the *CDH16*, *PPIA*, and *TBP* genes in the samples of degraded RNA from Caki-2 cells at a RIN value of 3 was 58%, 73%, and 27%, respectively, compared with the nondegraded samples, whereas the expression values for these genes in the RNA from renal tissue samples were only 9%, 8%, and 5%, respectively (Fig. 3, A and B).

RELATIVE CONCENTRATIONS OF miRNAs AND mRNAs IN NATIVE SAMPLES FROM NONPATHOLOGIC RENAL AND PROSTATE TISSUE

To exclude the possibility that our model of heat degradation at 80 °C does not correspond to the RNA degradation that occurs as samples are handled during collection, storage, and processing, we investigated whether the RIN-related effect on the gene expression data, as shown in the model experiments, is also evident in RNA samples with different RIN values isolated from tissue samples. For that purpose, we measured miR-155, miR-200c, and *CDH16*, *PPIA*, and *TBP* expression in samples of nonmalignant renal tissue after radical nephrectomy and measured miR-130b, miR-205, and *HIF1A*, *KLK3*, and *HPRT1* expression in nonmalignant samples of prostate tissue after radical prostatectomy (Fig. 4). To facilitate the statistical evaluation of the data, we used only nonmalignant tissue samples for this part of study because of their lower interindividual variation in gene expression compared

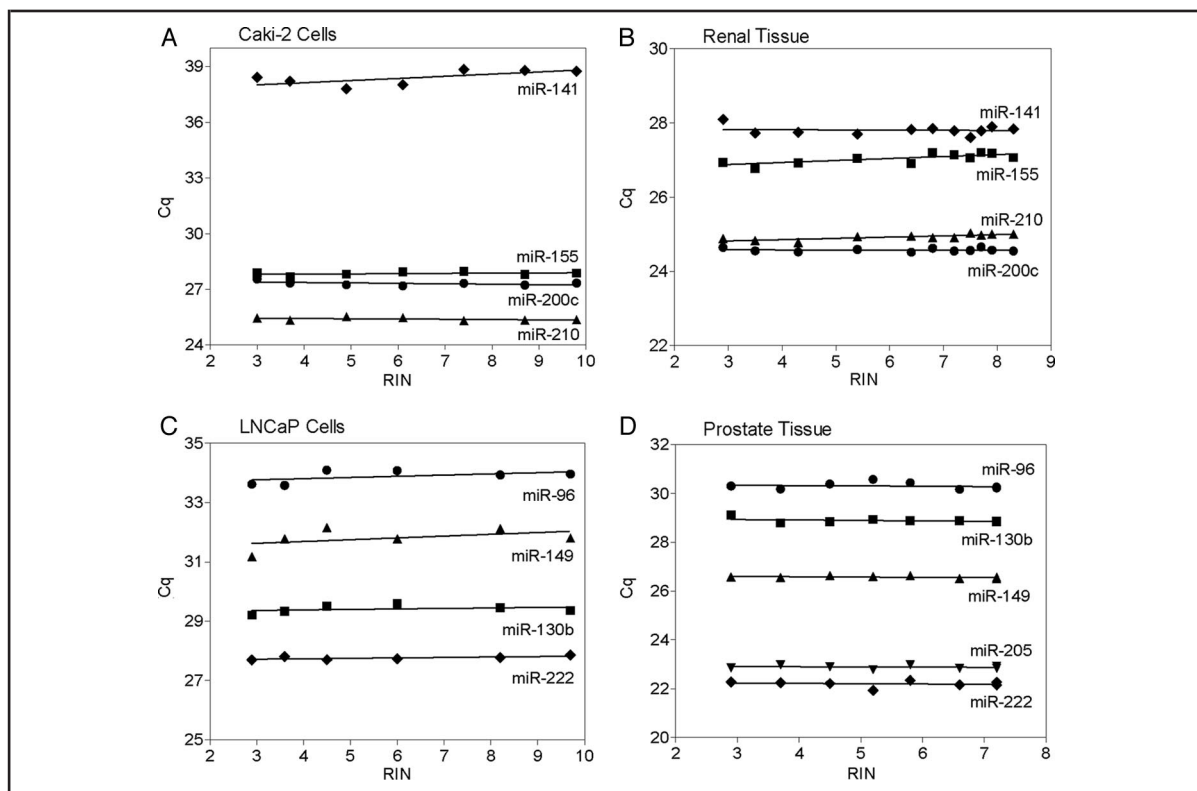


Fig. 2. Influence of RNA integrity on miRNA gene expression.

(A), miR-141, miR-155, miR-200c, and miR-210 in RNA samples from ccRCC cell line Caki-2. (B), miR-141, miR-155, miR-200c, and miR-210 in RNA samples from the renal tissue pool. (C), miR-96, miR-130b, miR-149, and miR-222 in RNA samples from PCa cell line LNCaP. (D), miR-96, miR-130b, miR-149, miR-205, and miR-222 in RNA samples from the prostate tissue pool. For further details, including regression line characteristics, 95% CIs of the slopes, and *P* values indicating significant deviations from 0, see Table 5 in the online Data Supplement.

with pathologic samples. Fig. 4 reveals that all mRNA concentration data were significantly dependent on RIN values. For example, the median expression values for the *PPIA*, *CDH16*, and *TBP* genes, as reflected in the RNA concentrations, in renal tissue samples with RIN values <4 decreased by 52%, 60%, and 65%, respectively, compared with the corresponding expression data for RNA samples with RIN values >6 (Fig. 4B). By contrast, miRNA concentrations in both tissue types were unrelated to the respective RIN values (Fig. 4, A and C).

Discussion

RNA integrity is the essential factor determining the accuracy of RT-qPCR measurements of mRNA transcripts, but there is scant information on the relationship between RNA integrity and miRNA stability. To the best of our knowledge, our study is the first systematic investigation of this issue.

Our results demonstrate that RNA integrity does not adversely affect the accuracy of RT-qPCR measurements of miRNAs in renal and prostate samples. The results of both the *in vitro*-degradation experiments (Fig. 2) and the experiments simulating *in vivo* degradation by measuring clinical samples characterized by different RIN values (Fig. 4, A and C) support this conclusion. The robust stability of miRNAs in degraded RNA samples enables miRNAs to be accurately quantified, even in degraded RNA samples for which mRNA measurements are no longer possible.

The intactness or integrity that characterizes the quality of isolated RNA can be influenced by several factors, such as the RNA source, the sampling technique, the isolation procedures, and storage conditions (19). Clinical samples in particular are subject to RNA degradation because of the often long interval between sample collection and their safe storage. In addition, RNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue, the material routinely ar-

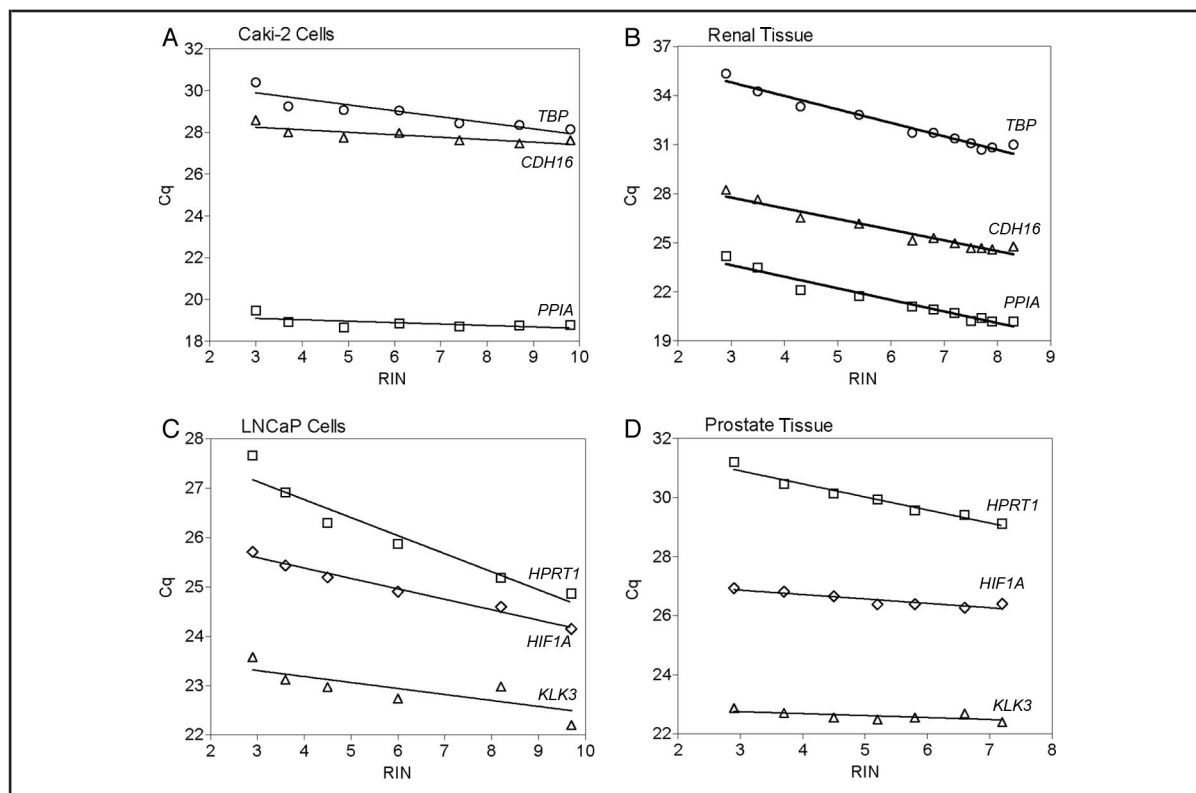


Fig. 3. Influence of RNA integrity on gene expression (mRNA).

(A), mRNAs for the *TBP*, *CDH16*, and *PPIA* genes in RNA samples from ccRCC cell line Caki-2. (B), mRNAs for the *TBP*, *CDH16*, and *PPIA* genes in RNA samples from the renal tissue pool. (C), mRNAs for the *HPRT1*, *HIF1A*, and *KLK3* genes in RNA samples from PCa cell line LNCaP. (D), mRNAs for the *HPRT1*, *HIF1A*, and *KLK3* genes in RNA samples from the prostate tissue pool. For further details, including regression line characteristics, 95% CIs of the slopes, and *P* values indicating significant deviations from 0, see Table 5 in the online Data Supplement.

chived in clinical diagnosis, is heavily degraded (28). Along with the consequences of erroneous downstream results, this characteristic is one of the main obstacles to the successful use of FFPE material for identifying and evaluating mRNA-based molecular markers. Thus, as briefly mentioned in the Introduction, the investigator should assess the extent of RNA degradation—the end point of all these detrimental factors that influence RNA intactness—before starting all downstream applications to avoid obtaining erroneous gene expression data. Corresponding recommendations to measure RNA integrity via the so-called RIN value or its equivalent were recently published in the MIQE guidelines (24).

RNA integrity constitutes the basis for all subsequent results and data interpretations. Establishing limits up to defined values were recommended for achieving reliable data in subsequent RT-qPCR measurements. For example, a RIN value of 5 was recommended as the lower limit for mammalian RNA, and a

RIN value of 7 has been recommended for bacterial samples (21, 29). In contrast to these limit-based approaches, corrective-based approaches for erroneously low data have been recommended to overcome the detrimental effect of RNA degradation (18, 30). Ho-Pun-Cheung et al. (18) developed a RIN-based corrective algorithm for RT-qPCR measurements for reducing possible errors due to RNA degradation from 100% to a mean error of <10%. Port et al. recommended a similar approach (30). Use of the *RN18S1* (RNA, 18S ribosomal 1) gene, which encodes 18S rRNA, as a reference gene and calculating the degree of degradation as the ratio of *RN18S1* expression in degraded samples relative to that in nondegraded samples allow erroneous gene expression data to be corrected for partially degraded RNA preparations. Other factors, such as the use of PCR primers that produce short amplicons, which are less sensitive to the interfering effect of RIN values, or the use of a special reverse-transcription priming strategy [e.g., random primers, target-specific

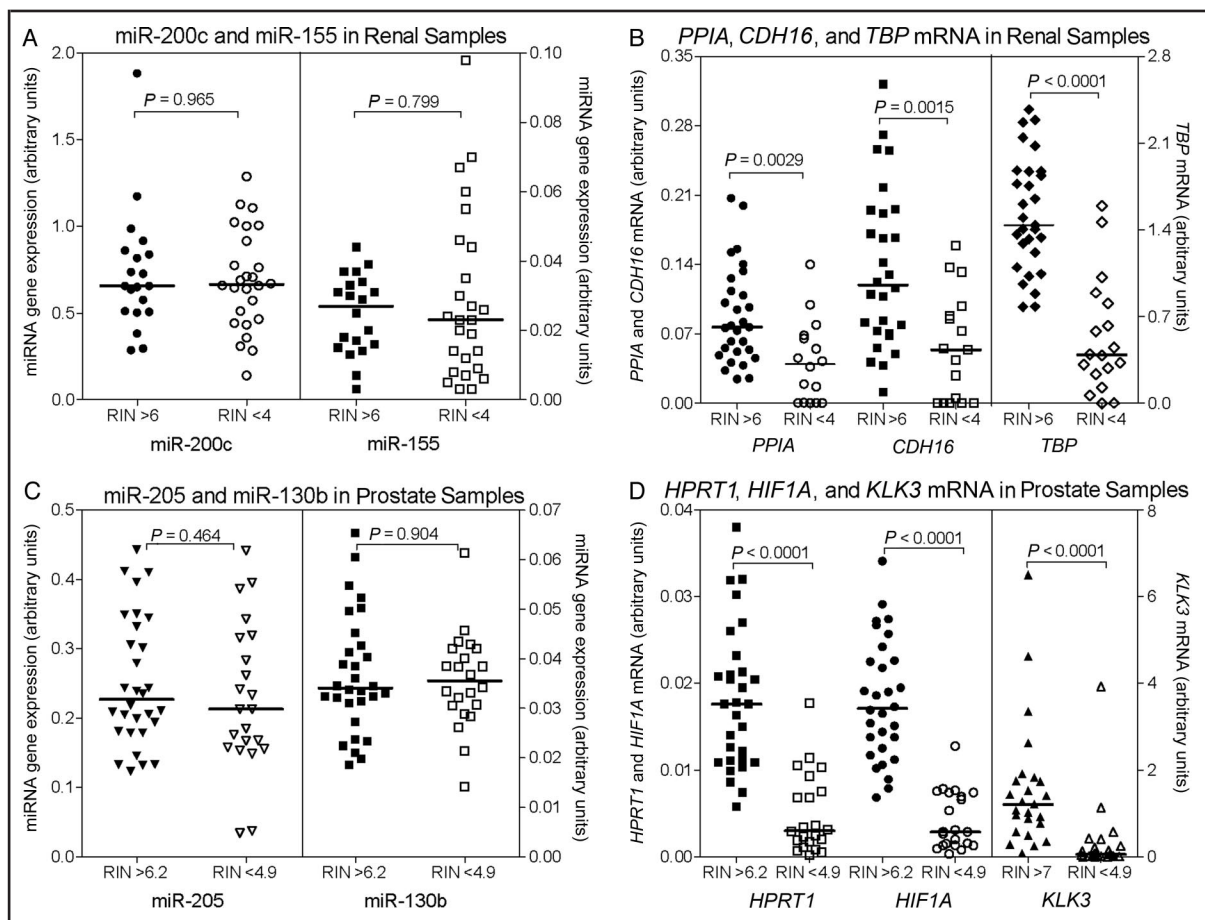


Fig. 4. Amounts of miRNAs and mRNAs in human nonpathologic, nonmalignant samples of renal and prostate tissues according to the RIN values of the isolated RNA.

The RIN study groups did not contain the same number of samples because of the limited availability of tissue material for testing. Amounts of miR-155 and miR-200c (A) and mRNAs encoded by the *PPIA*, *CDH16*, and *TBP* genes (B) in RNA samples with RIN values <4 (miRNA, n = 26; mRNAs, n = 18) and >6 (miRNAs, n = 20; mRNAs, n = 29) from renal tissue samples. miR-130b and miR-205 (C) and mRNAs encoded by the *HPRT1*, *HIF1A*, and *KLK3* genes (D) in RNA samples with RIN values <4.9 (n = 22) and >6.2 and >7.0 (n = 30 for miRNAs, *HPRT1* mRNA, and *HIF1A* mRNA; n = 25 for *KLK3* mRNA) from prostate tissue samples. Data are expressed as the median gene expression in arbitrary units. Statistical significance was evaluated with the Mann-Whitney U-test.

primers, or a mixture of random and oligo(dT) primers, as in our study] have also been proposed to reduce the detrimental effect of RNA degradation (19, 31–33). Our comparative mRNA measurements, however, have clearly shown that falsely low mRNA quantification can occur in degraded RNA preparations despite the use of short amplicons or a mixture of random hexamer and oligo(dT) primers and that the extent of the error also depends on the RNA source. For example, the reference genes *HPRT1* (127-bp amplicon) in prostate samples and *TBP* (227-bp amplicon) in renal samples were contrarily affected in cell and tissue samples, independently of their amplicon lengths in degraded RNA (Fig. 3). By contrast, the slopes of the re-

gression lines of the Cq values for these 2 reference genes were significantly different from those of the selected typical target genes (for further details, including regression line characteristics, 95% CIs of the slopes, and P values indicating significant deviations from 0, see Table 5 in the online Data Supplement). This result confirms that different mRNA species are affected differently in degraded RNA preparations (30). Consequently, the essential precondition that all mRNAs be identically impaired in degraded RNA samples for the correct normalization of target gene expression with respect to reference genes is not always guaranteed. These results also underline the fact that such corrections for RNA integrity via such approaches are

of limited value if small differences in transcripts should be reliably demonstrated.

The stability of miRNAs is clearly in contrast to the distinctly lower stability of mRNAs (Fig. 3). This miRNA stability, even in degraded RNA preparations, provides miRNA measurements with an essential analytical advantage over mRNA measurements. Thus, comparison of miRNA concentrations in tissue samples from different individuals is feasible if the miRNA results are related to the total RNA concentration. In this case, RNA concentrations in samples should not be less than the measurement limit of 100 ng/ μ L (34). The particular stability of miRNAs has already been assumed, given that miRNAs are only 20–22 nucleotides in length (28). Moreover, our results are indirectly supported by comparative miRNA and mRNA data for FFPE samples and fresh frozen samples [coefficients of determination (r^2) of 0.86–0.89 for miRNAs but only 0.28 for mRNA measurements (35)]. Although these investigators did not evaluate the association with individual RIN values and presented only characteristic RNA images showing the decrease in the 28S/18S ribosomal RNA ratio in FFPE samples compared with fresh frozen samples (28, 35), the conclusion regarding miRNA stability seems to be justified. Recently, Yu et al. (36) suggested, without giving further details, that the minimal RIN values for microarray and RT-qPCR miRNA-profiling experiments are 7.5 and 3.0, respectively. This method-dependent recommendation might be caused by the high fraction of fragmented RNA components in highly degraded RNA samples that interfere with the hybridization process but not with specific RT-qPCR measurements. That this methodologic particularity may also explain the miRNA decay described for human brain tissue samples detected by the microarray technique cannot be excluded (37).

By contrast, there are contradictory reports on the stability of miRNAs under identical storage conditions (38–40). Bravo et al. (40) observed high instability of miRNAs and their cDNA derivatives stored at -80°C as soon as 3 days after RNA isolation; however, 2 teams who reevaluated this result with the same isolation methods and storage conditions could not confirm this instability (38, 39). The necessity to maintain strict ribonuclease-free conditions in all miRNA-preparation steps has been pointed out (38). Under these conditions, RT-qPCR analyses of miRNAs showed stable data for RNA samples stored at -80°C over a period of 10 months (38). Other investigators have reported on the high stability of endogenous miRNAs in plasma samples. The comparative instability of exogenous purified miRNAs spiked into plasma samples has been explained as being due to endogenous nuclease activity in plasma (41, 42).

We conducted this study according to the MIQE guidelines, but we should mention some particular characteristics and possible limitations. First, the results relate to RT-qPCR measurements with TaqMan assays that use gene-specific looped primers; potential analytical interferences with such assays might preclude obtaining similar results with other methods. The use of the highly specific assay for mature miRNAs enables discrimination between miRNAs that differ by as little as a single nucleotide (17). Thus, only degraded mRNAs or other fragments that appear as free single strands with full-length nucleotide sequences identical to the mature miRNA sequences of interest could possibly be detected by this assay principle; We conducted a Blastn search of full-length mature miRNA sequences in the mRNA RefSeq database and found none of the sequences for our investigated miRNAs, with the exception of miR-155, which was located in its host gene [NR_001458.3; *MIR155HG* (MIR155 host gene (non-protein coding))]. Thus, the likelihood of the occurrence of such interfering sequences in our stability study seems to be rather small. Second, we used only 2 different tissue types (kidney, prostate) in our stability experiments. Although we did not observe tissue-specific miRNA stabilities, we cannot exclude that such variation occurs in other tissues. Third, the same limitation exists with respect to miRNAs themselves, because we tested the stabilities of only a few miRNAs. Although our results strongly suggest the high stability of miRNAs in general, other miRNAs with different stability behaviors are possible.

Despite these possible limitations, this systematic study is the first to demonstrate the robust stability of various miRNAs relative to mRNAs, at least in renal and prostate cell lines and tissues as assessed with TaqMan assays. This distinctive feature of miRNAs allows accurate RT-qPCR measurements of miRNA concentrations, even in the degraded RNA samples generally considered unsuitable for reliable mRNA measurements. Before this behavior of miRNA stability can be generalized, however, further studies of other miRNAs and tissue types, as well as of other measurement techniques, are required. Another interesting issue would be to investigate the stability of tissue-specific miRNAs in relation to the stability of their putative target genes. Moreover, these characteristics of robust stability could be helpful in introducing miRNAs as biomarkers into clinical practice, because miRNA data have provided more precise diagnostic results than mRNA profiles (10).

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting

or revising the article for intellectual content; and (c) final approval of the published article.

Authors' Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.

Research Funding: M. Jung, Sonnenfeld Stiftung, Berlin; K. Jung, Sonnenfeld Stiftung, Berlin, and Stiftung Urologische Forschung, Berlin; C. Stephan, Stiftung Urologische Forschung, Berlin; A. Schaefer, grant from Stiftung Urologische Forschung, Berlin; I. Steiner, grant from Stiftung Urologische Forschung, Berlin.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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