Identifying an appropriate endogenous control for real-time PCR of microRNAs in colorectal liver metastases: A comparison of 18s rRNA and small nuclear RNA RNU6B

CLEMENS FRANZ, SIBYLLE HARTMANN, ALEXIS ULRICH, GEORGIOS POLYCHRONIDIS, CHRISTOPH KAHLERT, FEE KLUPP* and MOHAMMED AL-SAEEDI*

Department of General, Visceral and Transplantation Surgery, Ruprecht-Karls-University Heidelberg, D-69120 Heidelberg, Germany

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Abstract. Selection of an appropriate housekeeping gene as an endogenous control is essential for obtaining reliable results in semi-quantitative real-time (RT-)PCR. However, the cellular composition varies according to the site of metastasis, resulting in differences among tissue compartments. Consequently, a suitable endogenous control for microRNA (miRNA) PCR may differ depending on the target organ, making individual evaluation of candidate controls necessary. In the present study, liver metastases were investigated, and RNU6B was compared with the well-established 18s ribosomal RNA (18s rRNA), commonly used in mRNA PCR, to determine which gene is more suitable as an endogenous control for miRNA analysis in colorectal liver metastases (CLM). A total of 79 patients with stage IV colorectal carcinoma who underwent liver resection were included. RNA, including miRNA, was extracted and analyzed using RT-PCR. The expression levels of RNU6B and 18s rRNA in CLM tissues and paired healthy liver tissues were assessed, and compared with the characteristics of a theoretically ideal endogenous control. The mean Cq values of RNU6B were significantly lower than those of 18s rRNA in both CLM tissues and corresponding healthy liver tissues ($P<0.001$). Pearson correlation analysis demonstrated a strong correlation between CLM and healthy liver tissues for RNU6B (correlation coefficient $\varrho=0.807$), whereas only a weak correlation was observed for 18s rRNA ($\varrho=0.398$). Furthermore, the slope and y-intercept of the regression line for RNU6B were closer to those of an ideal regression line than those for 18s rRNA. Clinical parameters, including sex, age, primary tumor localization and stage, and chronicity of liver metastases, did not significantly influence the expression of either RNU6B or 18s rRNA. Overall, RNU6B demonstrated greater conformity to the characteristics of an ideal endogenous control than 18s rRNA for miRNA PCR in CLM tissues. Based on these findings, RNU6B is recommended as an endogenous control for RT-PCR analysis of miRNAs in metastatic colorectal cancer.

Introduction

MicroRNAs (miRNAs) play critical roles in the development and progression of colorectal cancer (1), and their involvement in carcinogenesis and metastasis has been extensively investigated (2-4). In numerous studies, miRNA expression has been quantified using real-time (RT)PCR with the $^{\Delta\Delta Cq}$ method, microarray platforms and next-generation sequencing (NGS) (5,6). However, uncertainty remains regarding the optimal endogenous controls for PCR data normalization and accurate interpretation of these experiments (7,8).

It is widely accepted that no universal reference gene exists for miRNA PCR. Instead, combinations of multiple miRNAs are often required to achieve stable reference expression (9,10). Recently, the mean threshold cycle values of several miRNAs have been proposed as a universal normalizer (11). This global normalization strategy, which relies on mean expression without a specific endogenous control, is suitable for high-throughput miRNA profiling experiments involving large-scale datasets (12). However, small-scale miRNA RT-PCR studies require tissue-specific endogenous controls to normalize both biological variables, such as inter-individual and disease-associated differences, and technical factors, including inter-card variability between technical replicates (13).

Application of the $^{\Delta\Delta Cq}$ method requires both a target tissue, such as a primary tumor, and a reference tissue, such as healthy colon mucosa. In metastatic disease; however, the molecular differences between target and reference tissues are more pronounced, as these tissues typically do not originate from the same cellular background. Consequently, selection of an appropriate endogenous control becomes particularly critical when analyzing miRNA expression in metastatic cancers such

Correspondence to: Dr Clemens Franz, Department of General, Visceral and Transplantation Surgery, Ruprecht-Karls-University Heidelberg, Im Neuenheimer Feld 420, D-69120 Heidelberg, Germany
E-mail: clemens.franz@med.uni-heidelberg.de

*Contributed equally

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as colorectal liver metastases (CLM). To date, this issue has not been sufficiently investigated.

To identify suitable endogenous controls for miRNA analysis in CLM, a literature search was performed in the PubMed database using the following search terms: 'Polymerase Chain Reaction'[Mesh] OR 'Real-Time Polymerase Chain Reaction'[Mesh] AND 'MicroRNAs'[Mesh] AND 'Genes, Essential'[Mesh]. This search yielded ten relevant publications, indicating that the small nuclear (sn)RNA RNU6B was most frequently used as an endogenous control in miRNA PCR, whereas endogenous controls commonly applied in mRNA RT-PCR, such as 18s ribosomal RNA (18s rRNA), were rarely used. Although the present study focused on RNU6B and 18s rRNA, a similar approach could also be applied to other miRNAs.

18s rRNA consists of 1869 nucleotides and is a component of the 40S ribosomal subunit (https://www.ncbi.nlm.nih.gov/nuccore/NR_145820.1) (14). It has long been used as a classical endogenous control for RT-PCR following RNA extraction and cDNA synthesis in various human cell lines and is recognized for its low variability and reliability (15). In liver tissue, including alcoholic liver disease tissue, 18s rRNA is stably expressed and suitable as an endogenous control (16).

RNU6B, also known as RNU6-2 or U6-2, belongs to the U6 family of snRNAs and consists of 107 nucleotides (17). It is the most commonly used reference gene in miRNA PCR analyses across a wide range of carcinomas (11). RNU6B has been validated as a suitable endogenous control in multiple miRNA PCR studies, suggesting its potential applicability for miRNA expression analysis in CLM. However, its suitability in this specific context has not been systematically evaluated. To address this gap, the present study compared the performance of RNU6B and 18s rRNA as endogenous controls for miRNA RT-PCR normalization in CLM.

Materials and methods

Study population. A total of 79 patients with stage IV colorectal carcinoma who underwent partial liver resection for metastatic disease at the Department of General, Visceral and Transplantation Surgery, University Hospital of Heidelberg between 2009 and 2016, were included in the present study. The tissue samples were obtained during surgery within the operating room. Mostly, in case of major liver resections like hemi-hepatectomy, the resected liver specimen has enough healthy liver tissue as the metastases do not fill the whole volume of the resected liver. In case of atypical resection without sufficient amount of healthy liver in the resected sample or when there is a risk of compromising the resection margin, the surgeon takes a small specimen of healthy liver as a diagnostic sample without additional risks for the patient. Clinical parameters, including sex, age at the time of surgery, location and stage of the primary tumor, and chronicity of liver metastases, were retrospectively analyzed. The study protocol was approved by the Ethics Committee of the University of Heidelberg (ethics approval no. S-596/2015), and written informed consent was obtained from all patients.

RNA extraction and cDNA synthesis. Flash-frozen colorectal liver metastasis tissue samples and corresponding healthy liver tissue from all 79 patients were analyzed. Tissue identity was confirmed via histological examination. Samples were sectioned at 8 μm using a CM3050S cryostat microtome and stained with hematoxylin and eosin to distinguish healthy liver tissue from metastatic lesions. Tissue samples were homogenized using a TissueLyser II, and total RNA, including miRNA, was extracted using the miRNeasy Mini kit according to the manufacturer's instructions. RNA concentration and purity were assessed photometrically using a NanoDrop 2000 spectrophotometer and standardized to 500 ng/μl. The mean nucleic acid 260/280 ratio was 1.97 (95% CI, 1.93-2.00). Reverse transcription of RNA to cDNA was performed using the miScript II RT kit following the manufacturer's protocol.

RT-PCR. Semi-quantitative RT-PCR was performed using a LightCycler 480 II system with the miScript SYBR Green PCR kit and specific primers for RNU6B (Qiagen cat. no. MS00033740) and 18s rRNA (Qiagen cat. no. QT00199367). Amplification was conducted for 40 cycles, followed by melting curve analysis to confirm product specificity. The mean melting temperature was 77.22˚C (95% CI, 77.14-77.30˚C) for RNU6B and 86.16˚C (95% CI, 86.08-86.23˚C) for 18s rRNA. All samples were analyzed in technical duplicates, and mean Cq values were used for subsequent analyses. A theoretically ideal endogenous control was defined as one exhibiting identical Cq values in paired healthy liver and metastatic tissue from the same patient. Such a control would yield a linear regression with a slope of 1 and a y-intercept of 0 when plotting Cq values of healthy liver tissue against colorectal liver metastasis tissue. To simulate inter-sample variation, paired Cq values ranging from 15 to 26 were randomly generated using spreadsheet software, with identical values assigned to healthy liver and metastatic samples.

Statistical analysis. Statistical analyses were performed using SPSS software 31.0 (IBM Corp.). Normal distribution of continuous variables was assessed using the Shapiro-Wilk test. Descriptive statistics were expressed as mean ± standard deviation. Differences between 18s rRNA and RNU6B expression within the four groups were evaluated using a repeated measures ANOVA followed by the Bonferroni post hoc test. Differences between groups in terms of clinical parameters were evaluated using two-sided unpaired Student's t-tests or one-way analysis of variance, as appropriate. Associations between variables were assessed using Pearson's correlation analysis, and correlation coefficients were calculated. Dot plots with linear regression lines and 95% confidence intervals were generated. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical parameters of the study population. Among the 79 included patients, 46 were male (58.2%) and 33 were female (41.8%). The median age at the time of liver resection was 61 years, with an interquartile range of 53-68 years. The primary tumor was located in the colon in 50 patients (63.3%) and in the rectum in 29 patients (36.7%). Tumors were staged as T1, T2, T3 and T4 adenocarcinoma in 3 (3.8%), 7 (8.9%), 53 (67.1%), and 12 (15.2%) patients, respectively. With respect to metastatic timing, synchronous liver metastases were identified in 42 patients (53.2%), whereas metachronous liver metastases were observed in 37 patients (46.8%; Table I).

Table I. Clinical parameters of the study population, including sex, age, localization and stage of the primary tumor, and chronicity of liver metastases.

		RNU6B, liver		RNU6B, CLM		18s rRNA, liver		18s rRNA, CLM	
Parameter	N (%)	Cq-value	P-value	Cq-value	P-value	Cq-value	P-value	Cq-value	P-value
Sex			0.121		0.464		0.106		0.427
Male	46 (58.2)	18.92		19.18		26.99		26.10	
Female	33 (41.8)	18.17		18.73		26.39		25.78	
Age[a], years			0.842		0.803		0.954		0.194
≥61	41 (51.9)	18.65		18.92		26.75		25.71	
<61	38 (48.1)	18.56		19.07		26.72		26.23	
Localization			0.200		0.298		0.327		0.601
Colon	50 (63.3)	18.37		18.76		26.60		26.04	
Rectum	29 (36.7)	19.01		19.40		26.98		25.83	
Stage[b]			0.127		0.291		0.457		0.955
T1	3 (3.8)	21.10		21.56		25.36		25.99	
T2	7 (8.9)	18.06		18.17		26.71		25.62	
T3	53 (67.1)	18.43		18.92		26.64		25.94	
T4	12 (15.2)	19.04		19.10		27.00		25.70	
Chronicity			0.919		0.129		0.481		0.590
Synchronous	42 (53.2)	18.63		19.42		26.86		26.06	
Metachronous	37 (46.8)	18.58		18.51		26.60		25.85	

[a]The median age was 61.0 years (range, 35-88 years). [b]One-way ANOVA. The primary colorectal tumor stage could not be determined in 4 cases of metachronous metastases because the primary tumor resection had been performed at a different hospital and data for T stage could only be obtained for 75 patients. The absolute number of patients (N) and relative proportion (%) are shown. For each dichotomous parameter, corresponding mean Cq values were compared using a two-sided unpaired Student's t-test for RNU6B and 18s rRNA and liver and CLM. These parameters did not significantly affect RNU6B and 18s rRNA expression. 18s rRNA, 18s ribosomal RNA; CLM, colorectal liver metastasis.

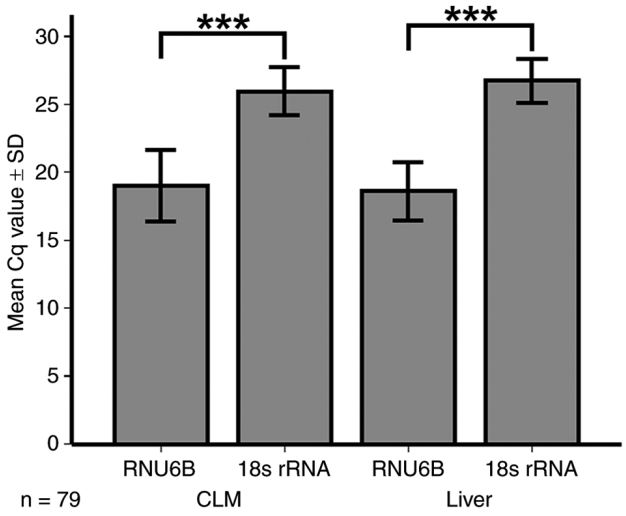

Figure 1. Mean Cq value ± SD for RNU6B and 18s rRNA in CLM and corresponding healthy liver tissues. Cq values for RNU6B were significantly lower than those for 18s rRNA in both CLM and corresponding healthy liver tissues (***P<0.001; repeated measures ANOVA followed by the Bonferroni post hoc test). 18s rRNA, 18s ribosomal RNA; CLM, colorectal liver metastasis; SD, standard deviation.

RT-PCR results and generated endogenous control. RT-PCR was performed to quantify RNU6B and 18s rRNA expression in CLM and corresponding healthy liver tissue. The mean Cq values of RNU6B were significantly lower than those of 18s rRNA in both CLM and healthy liver tissue (18.99 in CLM and 18.61 in healthy liver tissue for RNU6B; 25.97 in CLM and 26.74 in healthy liver tissue for 18s rRNA; both P<0.001; Fig. 1). The results of the repeated measures ANOVA followed by the Bonferroni post hoc test demonstrated a not significant difference between CLM and healthy liver tissue for RNU6B (P=0.185) and a significant difference for 18s rRNA expression (P=0.002; Fig. 1).

The randomly generated endogenous control exhibited a mean Cq value of 20.43 and identical values for both healthy liver and metastatic tissue. As the x- and y-axis values were equal for all samples (n=79), Pearson's correlation analysis revealed a significant correlation (P<0.001), a correlation coefficient of 1.0 and a regression line defined as y=1·x+0 (Fig. 2A).

Pearson's correlation analysis between CLM and healthy liver tissue demonstrated significant correlations for both RNU6B and 18s rRNA (P<0.001). A strong correlation was observed for RNU6B (ϱ=0.807), whereas a weak correlation was found for 18s rRNA (ϱ=0.398; Fig. 2B and C). The regression equation was y=1·x+0.35 for RNU6B and y=0.43·x+14.44 for 18s rRNA.

For each primer and tissue type, potential influences of clinical parameters were statistically evaluated. No significant differences in RNU6B or 18s rRNA expression were detected with respect to sex, age, tumor localization, tumor stage, or chronicity of metastases (all P>0.05; Table I). The performance characteristics of RNU6B and 18s rRNA were then compared with those of a theoretically ideal endogenous control. RNU6B matched six criteria of the ideal control, whereas 18s rRNA matched three. Only two criteria deviated slightly from the ideal for RNU6B, while five criteria showed substantial deviation for 18s rRNA (Table II).

Discussion

In the present study, the suitability of 18s rRNA and RNU6B as endogenous controls for small-scale miRNA RT-PCR in CLM tissue was confirmed. The results demonstrated that RNU6B closely approximated the characteristics of an ideal endogenous control, including high expression levels, strong correlation between healthy liver tissue and CLM tissue, and comparable length between the reference target and the amplified miRNA products.

The importance of miRNAs in screening, diagnosis and as potential therapeutic targets in colorectal cancer has increased substantially over recent decades (1,4). However, reliable and reproducible results from molecular techniques such as RT-PCR depend critically on the use of appropriate endogenous controls for normalization (18). Although multiple mathematical tools have been developed to identify stable reference genes, none currently serve as a universal gold standard (11). In small-scale RT-PCR experiments, a practical approach is to compare a candidate endogenous control with a well-established reference gene to determine relative suitability.

Although 18s rRNA is a validated endogenous control for mRNA RT-PCR and effectively compensates for differences in RNA input with low variability (15), it is suboptimal for small-scale miRNA PCR analyses. This limitation is largely due to fundamental differences between 18s rRNA and miRNAs, including target length, amplification kinetics and expression abundance. Ideally, the endogenous control should closely resemble the target miRNA in these properties to ensure accurate normalization (7,19,20).

Comparison of RNU6B and 18s rRNA expression in CLM and corresponding healthy liver tissue revealed that RNU6B exhibited consistently lower Cq values than 18s rRNA, indicating higher expression levels despite generally low expression levels of small RNAs (21). This observation is likely attributable to the use of RNA extraction, reverse transcription and amplification protocols optimized specifically for miRNAs rather than for longer RNA species.

In theory, an ideal endogenous control would display perfectly correlated Cq values between paired tissues, producing a regression line with a slope of 1 and a y-intercept of 0. Although this theoretical model does not account for biological variability and is not fully achievable in practice, it provides a useful benchmark. In the present study, the regression line comparing RNU6B Cq values between healthy liver tissue and CLM closely approximated this ideal model, with a slope near 1 and a y-intercept close to zero. By contrast, 18s rRNA demonstrated substantially greater deviation from these parameters.

As disease states can influence the expression of genes commonly used for normalization (17,22), it is essential to evaluate the impact of clinical and pathological factors on candidate endogenous controls. Gene expression in colorectal cancer tissues may be affected by sex, age, primary tumor localization, and chronicity of metastatic disease. In the present study, none of these variables significantly influenced the expression of either RNU6B or 18s rRNA in CLM.

The findings indicated that RNU6B is a suitable endogenous control for small-scale miRNA RT-PCR analyses in CLM tissue. By contrast, another U6 family member, RNU6, has been reported to be unsuitable as an endogenous control

Table II. Performance characteristics of a theoretically ideal EC for analysis of microRNAs using semi-quantitative real-time PCR and the ΔΔCq method in comparison with RNU6B and 18s rRNA.

Parameter	Ideal EC	RNU6B	18s rRNA
Ubiquitously expressed in investigated tissues	Yes	Yes[a]	Yes[a]
High RNA content/low Cq value	Yes	Yes[a]	No[b]
Correlation between tissues (ϱ)	Linear (1.0)	Very strong (0.807)	Weak (0.398)
Regression line of Cq correlation			
Slope	1.00	1.00[a]	0.43[b]
y-intercept	0.00	0.35[a]	14.44[b]
No influence of clinical parameters on Cq value	Yes	Yes[a]	Yes[a]
Similar length of target	Yes	Yes[a]	No[b]
Nucleotides	25-100	107	1,869
Similar length of PCR product	Yes	Yes[a]	No[b]
T_m	$T_{m, target}=T_{m, EC}$	77.22˚C	86.16˚C
Specificity of primer for target	Yes	Yes[a]	Yes[a]
Peak in melting curve	Single	Single	Single

[a]Consistent with ideal EC. [b]No match with ideal EC. 18s rRNA, 18s ribosomal RNA; EC, endogenous control; T_m, melting temperature.

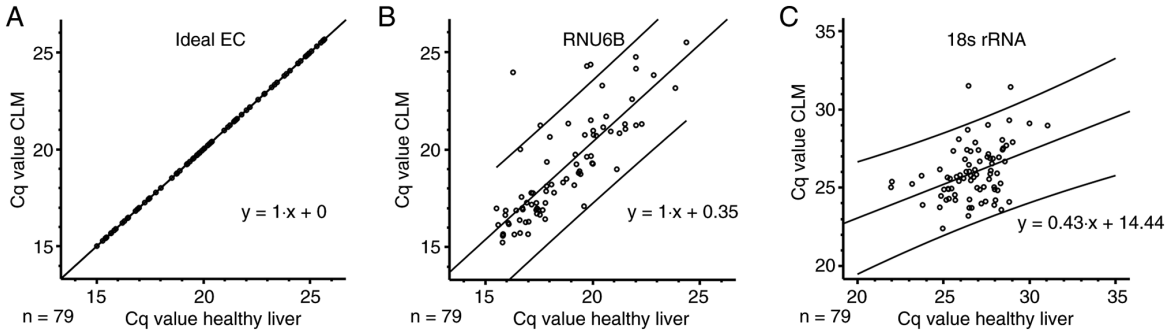

Figure 2. Correlation of Cq values between CLM and corresponding healthy liver tissues. Correlations are shown for (A) an ideal EC, (B) RNU6B and (C) 18s rRNA. Compared with 18s rRNA, RNU6B more closely approximated the ideal endogenous control. The ideal regression line is shown in (A) (y=1·x+0), whereas (B and C) present regression lines with the 95% CI for RNU6B (y=1·x+0.35) and 18s rRNA (y=0.43·x+14.44), respectively. 18s rRNA, 18s ribosomal RNA; CLM, colorectal liver metastasis; EC, endogenous control.

in RT-PCR studies of circulating miRNAs in serum and plasma (23), a finding also observed in patients with sepsis or liver fibrosis, where RNU6B expression was dysregulated (24). Although these reports appear to contradict the present findings, RNU6 and RNU6B are distinct genes and should not be directly compared. Moreover, miRNA analyses of circulating exogenous miRNAs differ substantially from analyses of endogenous tissue miRNAs, particularly with respect to RNA yield, RNase activity and molecular context, such as protein-bound or vesicle-encapsulated miRNAs (25,26).

Notably, evaluating a limited number of candidate endogenous controls restricts the generalizability of the conclusions. Additionally, a theoretical model with ideal assumptions cannot fully reflect reality due to the lack of biological variability. Nevertheless, this approach represents a practical alternative to more complex normalization strategies involving multiple reference genes and computational tools such as geNorm, NormFinder or BestKeeper (27-29). For large-scale RT-PCR studies, normalization strategies incorporating multiple reference genes are often preferable (9).

In conclusion, the present study demonstrated that RNU6B shares key characteristics with an ideal endogenous control for miRNA RT-PCR analysis in CLM and corresponding healthy liver tissue. Although these findings support the use of RNU6B in this specific context, they cannot be extrapolated to other tumor entities without further validation. Additional studies examining other metastatic sites are warranted to improve miRNA normalization strategies in metastatic disease. Based on the present findings, RNU6B is recommended as an endogenous control for small-scale miRNA RT-PCR experiments in metastatic colorectal cancer tissues. Notably, the suitability of any miRNA reference gene should be evaluated individually for each RT-PCR experimental setup.

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Availability of data and materials

The data generated in the present study are not publicly available due to privacy and ethical reasons, but may be requested from the corresponding author.

Authors' contributions

CF was responsible for the conception and design of the study, acquisition, analysis and interpretation of data, and drafting and revising the manuscript. SH and AU were also responsible for acquisition, analysis and interpretation of data, and drafting and revising the manuscript. GP, CK, FK and MAS were responsible for study design, interpretation of data, and critical revision and editing of the manuscript. CF, SH and AU confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the University of Heidelberg (approval no. S-596/2015; Heidelberg, Germany). Written informed consent was obtained from all participants. The study was performed in accordance with The Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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