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Identification and performance evaluation of housekeeping genes for microRNA expression normalization by reverse transcription-quantitative PCR using liquid-based cervical cytology samples

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Abstract. Screening for cervical cancer by cytology has been effective in reducing the worldwide incidence and mortality rates of this disease. However, a number of studies have demonstrated that the sensitivity of conventional cervical cytology may be too low for detection of cervical intraepithelial neoplasias (CIN). Therefore, it is important to incorporate more sensitive molecular diagnostic tests that could substantially improve the detection rates and accuracy for identifying CIN lesions. MicroRNAs (miRNAs) are a class of small non-coding RNAs with the potential to provide robust non-invasive cancer biomarkers for detecting CIN lesions in liquid-based cervical cytology (LBC) samples. At present, there is no consensus on which are the best housekeeping genes for miRNA normalization in LBC. The present study aimed to identify housekeeping genes with consistent and reproducible performance for normalization of reverse transcription-quantitative PCR (RT-qPCR) expression analysis of miRNA using LBC samples. The present study firstly selected six potential candidate housekeeping genes based on a systematic literature evaluation. Subsequently, the expression levels of microRNAs U6, RNU-44, RNU-47, RNU-48, RNU-49 and hsa-miR-16 were measured in 40 LBC samples using RT-qPCR. The stability of each potential housekeeping gene was assessed using the NormFinder algorithm. The results revealed that U6 and RNU-49 were the most stable genes among all candidates requiring fewer amplification cycles and smaller variation across the sample set. However, RNU-44, RNU-47, RNU-48 and hsa-miR-16 stability exceeded the recommended housekeeping value suitable for normalization. The findings revealed that U6 may be a reliable housekeeping gene for normalization of miRNA RT-qPCR expression analysis using LBC samples.

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Abbreviations: CC, cervical cancer; miRNA, microRNA; RT-qPCR, reverse transcription-quantitative PCR; Cq, cycle quantification; CIN, cervical intraepithelial neoplasia; HFS, healthy female subjects

Key words: cervical cancer, miRNA expression, liquid-based cytology, housekeeping genes, reverse transcription-quantitative PCR

Introduction

Cervical cancer (CC) is the fourth most common cancer among women, with an estimated 527,600 new cases and 265,700 deaths worldwide (1). In developed countries, the incidence and mortality rates have decreased significantly in the past decades. In contrast, the burden from CC remains high in developing and underdeveloped countries due to continuing challenges implementing effective prevention and control programs. Difficulties in less developed countries include barriers to accessing health care services, which are compounded by inadequate cytological examination, usually involving screening with the low coverage of Papanicolaou (Pap) test (2). To implement additional strategies for improving CC screening, some programs have focused on introducing innovative molecular diagnostic tests such as the molecular HPV testing that could provide more sensitive and specific detection of precursor lesions once validated.

MicroRNAs (miRNAs) are small non-coding RNAs (19 to 24 nucleotides) involved in the post-transcriptional regulation of gene expression (3), where they play a critical role in several cellular processes, such as proliferation, cell growth and apoptosis (4). Many studies have reported aberrant expression of miRNAs in cancer (5), suggesting that these molecules could be used as potential tumor biomarkers.

Indeed, a recent systematic review identified differentially expressed miRNAs in precursors cervical lesions and CC that could be associated with tumor progression (6). However, most analyses of miRNA expression in CC have used tumor tissue samples obtained from invasive procedures that cause patient discomfort, such as cervical tissue biopsies or surgery (7). The development of minimally invasive liquid biopsy cytology (LBC) based on miRNA expression (7) is a new approach to identify non-invasive biomarkers for early diagnosis, monitoring response to therapies and for tumor progression (7-9). LBC are considered as an accurate and promising low-cost method for clinical practice but the standardization of miRNA expression analysis remains a major challenge. Thus, standardization of this detection technique is fundamental for the reproducible use of miRNA biomarkers in clinical practice.

Reverse transcription-quantititative PCR (RT-qPCR) is a robust technique frequently used in the diagnosis of many neoplasms and infectious diseases due to its high sensitivity and specificity (9,10). Analysis of miRNAs is considered an important new biomarker because miRNAs are specific and stable in diverse types of clinical samples. However, identification of a constitutively expressed housekeeping genes for adequate normalization of miRNAs expression analysis is a crucial step for better accuracy with this technique. The use of housekeeping genes as endogenous control is the most common method for normalizing RT-qPCR data for miRNA expression (11). Housekeeping genes are internal reaction controls used to gene expression normalization of distinct miRNAs, and they can have different isoforms. For a gene to be considered a reliable housekeeping transcript, it needs to meet some stringent performance criteria (12), such as minimal expression variability between tissues and physiological states of the organism. Moreover, the normalization control being used must faithfully measure any technical variability resulting from differences in the quantity or quality of genetic material being tested (13). Above all else, the most important of these criteria is that the pattern of expression of the normalizer does not interfere or produce artifactual changes in the test samples. Satisfying these basic conditions are the essential properties of a good housekeeping gene for transcript normalization from specific biological samples of interest. The identification of suitable housekeeping genes is a crucial step for deriving reproducible results when investigating the differential expression of miRNAs. The use of unreliable normalization control genes can lead to an incorrect estimate of the expression levels of miRNAs of interest (14,15). For this reason, the choice of appropriate housekeeping genes for normalizing the expression of miRNAs analysis using LBC is an important issue to be solved. No housekeeping gene is unique and constitutively expressed in all sample types, as well as different types of diseases in all experimental designs, which indicates that the stability of housekeeping gene expression should be checked rigorously (16,17).

This is the first study to evaluate control housekeeping genes for miRNA RT-qPCR data normalization in LBC cervical samples. Since there is little consensus on the best choice of normalizers, we performed a literature review to identify housekeeping genes most commonly used in miRNA RT-qPCR data normalization. In addition, we evaluated their relative expression levels in LBC samples from patients who underwent routine cervical cancer screening.

Materials and methods

Study design. In order to select suitable housekeeping genes, we conducted a systematic two-phase analysis including an initial exploratory review of the literature, followed by a laboratory evaluation phase of selected genes (Fig. 1).

Phase 1, exploratory phase. We first performed a *PubMed* systematic literature review to identify candidate housekeeping genes for miRNA normalization in RT-qPCR using the following keywords: Cervical cancer; miRNA expression, RT-qPCR. Inclusion criteria were defined as follows: i) Original articles; ii) the language in which the article should be published was English; iii) studies that only used precursor lesions or CC samples; iv) studies that performed miRNA expression for RT-qPCR. We found a total of 109 articles, which were available at the end of March 2018. Of these, 70 studies (10-79) were eligible for inclusion in our selection of candidate housekeeping genes (Table I).

Phase 2, evaluation phase. All candidate housekeeping genes identified by our literature review were selected for expression analysis by RT-qPCR and tested in the LBC samples from 5 CC (5), 20 CIN (5 CIN3; 5 CIN2 and 10 CIN1) and 15 healthy women (HSF-without CIN). We considered a housekeeping gene to be suitable for normalization purposes when it was stably expressed across all samples independently of the histological condition, and when the cycle quantification (Cq) values did not exceed 35. Finally, the best housekeeping gene was evaluated using the NormFinder algorithm, which is software designed to identify the optimal normalization gene among a set of candidates (80).

Study population and sample collection. We analyzed a total of 40 LBC samples randomly obtained from women who had undergone routine colposcopy in the Department of Prevention of the Barretos Cancer Hospital in 2014. All samples were collected immediately before colposcopy and preserved in ThinPrep™ Pap test (Hologic) for subsequent molecular analyses. ThinPrep™ samples were classified into five groups: HFS; low-grade CIN (CIN1); high-grade CIN (CIN2 group or CIN3 group); and CC. All CIN- and CC-histological diagnoses from women who presented with suspicious/abnormal areas during colposcopy were subsequently confirmed by analyses of tissue samples collected for the Department of Pathology of the Barretos Cancer Hospital using a colposcopy-guided cervical biopsy.

RNA isolation. ThinPrep™ samples were manually washed to remove the buffered preservative solution and to lyse blood cells, which could inhibit downstream molecular analyses. Total RNA was performed using the RecoverAll Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. The purity of total RNA was evaluated by NanoDrop® Spectrophotometer v3.7 (Thermo Fisher Scientific).

RT-qPCR. Considering that the focus of this study is LBC cervical samples we do not use housekeeping genes to analyze

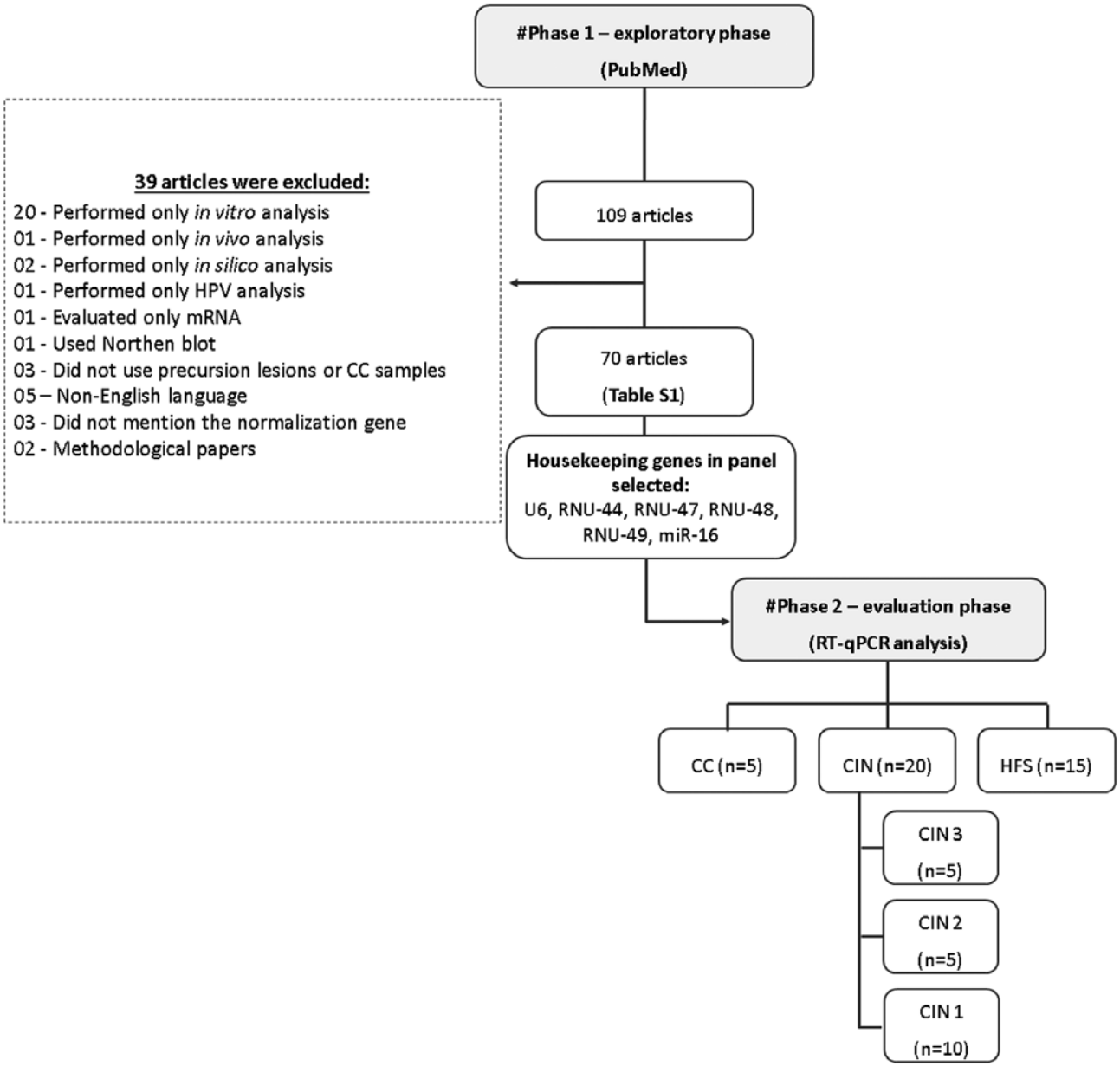

Figure 1. Flowchart depicting the exploratory and evaluation phases of selection of suitable housekeeping genes for normalization. CC, cervical cancer; CIN, cervical intraepithelial neoplasia; HFS, healthy female subjects; RT-qPCR, reverse transcription-quantitative PCR.

the expression of these miRNAs in tissue samples. To perform RT-qPCR reactions we used TaqMan microRNA assays (Thermo Fisher Scientific) using LBC cervical samples. Initially, a target-specific stem-loop reverse transcription RT-PCR was performed using a High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific), following the protocol provided by the manufacturer. Briefly, for each sample 10 ng of total RNA was reverse transcribed using miRNA-specific primers and TaqMan Assays (Table SI) in a 15 μl reaction volume for 30 min at 16˚C, 20 min at 42˚C and 5 min at 85˚C. All RT-PCR reactions were performed using the Proflex™ 3x32-well PCR system (Thermo Fisher Scientific). Then 2 μl of the reverse transcription products (cDNA) was amplified in the QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific) using the TaqMan Universal PCR Master Mix II (Thermo Fisher Scientific), according to the manufacturer's protocol. All RT-qPCR reactions were performed in triplicate using Taqman probes. The PCR protocol comprised 40 cycles of 2 min at 50˚C, 10 min at 95˚C, 15 sec at 95˚C and 1 min at 60˚C. The Threshold Cycle (Cq) values were determined using the same threshold setting and analyzed according to a previously reported method (81).

NormFinder-based stability analysis. Comparison between expression values was performed using NormFinder algorithm (80), an ANOVA model-based approach which estimates intragroup and inter-group expression variations in order to calculate stability values for each candidate gene (18). We used exponentially transformed Cq values (2^{-Cq}) as input data in the NormFinder software, which ranks genes based on their expression stability in a given sample set with a specific experimental design. The lowest value calculated by the software indicates the most stable endogenously expressed gene for optimal normalization.

Table I. Bibliographic survey of reference genes.

Author, year	Reference gene	Sample	(Refs.)
Yao *et al*, 2013; Yang *et al*, 2015; Yu *et al*, 2014; Wang *et al*, 2013; Yin *et al*, 2015; Wang *et al*, 2015; Song *et al*, 2015; Li *et al*, 2015; Wei *et al*, 2012; Chen *et al*, 2013; Yue *et al*, 2011; Zhou *et al*, 2016; Chen and Liu, 2016; Sun *et al*, 2016; Zhang *et al*, 2016; Xiao *et al*, 2014; Yi *et al*, 2016; Cheng *et al*, 2016; Sun *et al*, 2017; Yu *et al*, 2013; Yang *et al*, 2015; Qin *et al*, 2015; Sun *et al*, 2017; Chandrasekaran *et al*, 2016; Lin *et al*, 2017; Azizmohammadi *et al*, 2017; Jin *et al*, 2017; Liu and Ni, 2018; Zhou *et al*, 2017; Song *et al*, 2017; Zhao *et al*, 2017; He *et al*, 2017; Li *et al*, 2017; Hu *et al*, 2017; Luo *et al*, 2017; Zhang *et al*, 2018; Li *et al*, 2017; Zhao *et al*, 2017; Tao *et al*, 2017	U6	Tissue	(10-48)
Gocze *et al*, 2015; Zhang *et al*, 2013; Chen *et al*, 2014; Wang *et al*, 2015; Zeng *et al*, 2015; Ma *et al*, 2015; Zheng *et al*, 2015; Zhang *et al*, 2016	U6	FFPE	(49-57)
Zhao *et al*, 2013; Jia *et al*, 2015; Jiang *et al*, 2017; Zhou *et al*, 2017	U6	Serum	(58-61)
Yu *et al*, 2012	U6	Blood	(62)
Chen *et al*, 2013; Hu *et al*, 2017; You *et al*, 2015; Luo *et al*, 2015; Liu *et al*, 2015; Xie *et al*, 2015; Shen *et al*, 2013; Huang *et al*, 2012	RNU6B	Tissue	(19,43,63-68)
Xing *et al*, 2013; Lee *et al*, 2014	RNU6B	FFPE	(69,70)
Ivanov *et al*, 2018	U6	Air-dried cervical PAP smears	(71)
Gocze *et al*, 2015; Yu *et al*, 2016; Hao *et al*, 2016	5S	FFPE	(49,72,73)
Shen *et al*, 2013	18S	Tissue	(66)
Sun *et al*, 2017; Nagamitsu *et al*, 2016	miR-16	Serum	(74,28)
Kogo *et al*, 2015; Lajer *et al*, 2012	RNU44	Tissue	(75,76)
Chen *et al*, 2013	RNU44	Serum	(19)
Huang *et al*, 2014; Myklebust *et al*, 2011	RNU48	Tissue	(77,78)
Chen *et al*, 2013	RNU48	Serum	(19)
Sharma *et al*, 2016	miR-127	Tissue	(79)

FFPE, formalin-fixed paraffin-embedded.

Statistical analysis. All variables were presented using mean values and standard deviation (SD). ANOVA with a Bonferroni post hoc test and the Kruskall-Wallis tests were used to compare the mean values of continuous variables across the histologic groups. P-values of <0.05 were considered statistically significant. All statistical analyses were performed with SPSS for Windows, v.21.0 (IBM Corporation). All graphs was expressed just descriptive analysis data.

Results

We selected six candidate housekeeping genes based on their expression profile across the reviewed studies (Table I): U6 (U6 small nuclear RNA); miR-16 (hsa-microRNA-16); RNU-44 (SNORD44 small nucleolar RNA); RNU-48 (SNORD48 small nucleolar RNA); RNU-47 (SNORD47 small nucleolar RNA); and RNU-49 (SNORD49A small nucleolar RNA). Most of the candidate genes have previously been described as housekeeping transcripts for miRNA normalization for expression quantification using different types of biological samples, such as fresh tissue biopsies, formalin-fixed paraffin-embedded (FFPE) tissues, serum or plasma, and air-dried cervical smears. Of the six selected housekeeping genes, only miR-16 has previously been used as an endogenous control for LBC expression analysis (28,74).

The technical performance of six selected candidate housekeeping genes as normalization controls was investigated using 40 LBC samples. We found that U6 and RNU-49 had the lowest Cq value variation among the six tested candidate housekeeping genes (Fig. 2). Furthermore, both genes amplified more efficiently than the other candidates and required fewer amplification cycles to achieve Cq values above background fluorescence levels. The lower Cq value for U6 (21,81) indicated that their expression levels and PCR efficiencies required fewer cycles of amplification to reach the detection threshold. In addition, we found that the U6 gene was more uniformly expressed in LBC samples than the other candidate housekeeping genes. In contrast, more cycles of amplification were required (Cq values >35) for RNU-44, RNU-47, RNU-48 e miR-16, indicating that these genes might

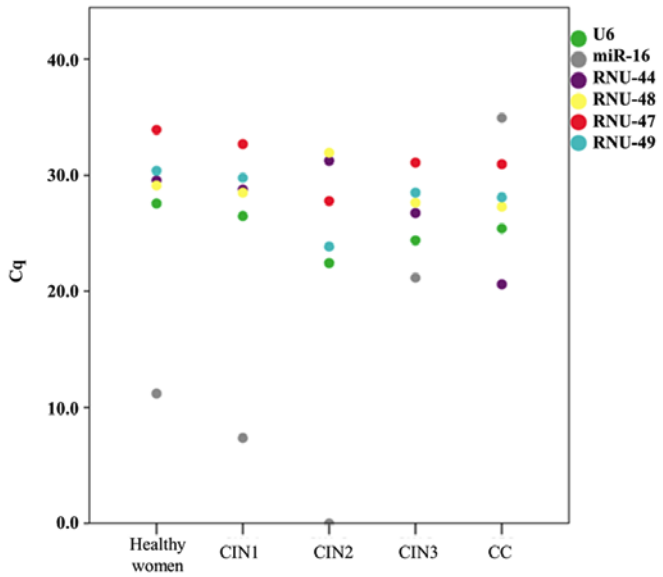

Figure 2. Distribution of mean Cq values according to histologic groups. CIN, cervical intraepithelial neoplasia; Cq, cycle quantification; CC, cervical cancer.

not be suitable housekeeping genes for normalization using LBC samples.

Candidate housekeeping gene expression according histological groups. To assess whether the candidate housekeeping genes were differentially expressed in varying histologic conditions, we compared the mean Cq values of the candidate housekeeping genes obtained from each of five histologic groups (Table II). We found no significant differences in the expression of U6 (P-value, 0.06) and RNU-49 (P-value: 0.128) across all groups, supporting their stable performance and potential as robust endogenous controls in RT-qPCR normalization using LBC samples. We did not find a significant association for RNU-47 (P-value, 0.064) and this gene required a greater number of amplification cycles across the experimental groups, especially in LBC samples from patients HFS (34.16±2.85). We also observed significant differences for miR-16 (P-value, 0.045); RNU-44 (P-value, 0.004); and RNU-48 (P-value, 0.022), indicating that these genes can be differentially expressed in LBC samples from patients with different cervical histology and that such variation could lead to inconsistent normalization. Indeed, miR-16 exhibited the most variable expression across the groups (Fig. 3), ranging from no amplification at all in LBC samples from CIN2 patients to low levels in CIN1 patients (7.35±15.51). Furthermore, RNU-44 and RNU-48 were more abundant in LBC samples from CC patients (mean Cq values: 20.60±11.60 and 27.27±2.30, respectively) in comparison to other histologic groups indicating that they would likely bias expression values.

Expression stability analysis. We further analyzed the stability values of each candidate housekeeping gene using the NormFinder algorithm. We found that among the six candidates, U6 was the most stable gene (stability value, 0.856), followed by RNU-49 (0.929) (Fig. 4). In contrast, the other candidate housekeeping genes presented inadequate stability values, ranging from 2.375 to 12.901. These findings suggested that U6 and RNU-49 were the best housekeeping genes in LBC samples, whereas miR-16, RNU-44, RNU-47, and RNU-48 should not be considered suitable for use as endogenous controls for RT-qPCR normalization.

Discussion

There have been several studies investigating the utility of miRNA in translational research, considering deregulated expression in diverse diseases, variation in tissue-specific distribution and the overall stability of miRNA in different clinical samples (19-22). Indeed, there is emerging evidence demonstrating the feasibility of using miRNAs as non- or minimally invasive diagnostic biomarkers in cancer. For instance, Rossi *et al* (82) evaluated a five-miRNA expression signature developed for thyroid lesions using fine needle aspiration cytology (FNAC). Their analysis suggested miR-375 as a promising preoperative biomarker for distinguishing benign from malignant follicular neoplasms. In another study, Kottaridi *et al* (83) designed a panel of seven overexpressed miRNAs for use in histologically confirmed LBC malignant endometrial samples to discriminate between non-malignant and malignant specimens and to identify any samples with inadequate RNA. There are many studies that have reported promising molecular approaches to LBC samples for clinical laboratories (84-86). None of the studies to date have focused on evaluating miRNAs in LBC cervical samples, which could be considered an important minimally invasive approach for cancer detection by miRNA expression data. For this reason, RT-qPCR is now one of the most commonly used new methods for the evaluation of miRNA expression due to its high sensitivity and reproducibility (87,88).

Since reliable normalization is fundamental to RT-qPCR, there is a need to choose a suitable gene for use as an endogenous control in order to obtain an accurate miRNA expression and to ensure consistency. The selection of housekeeping genes as normalizers for miRNA has relied on choosing from distinct miRNAs and other small RNAs, such as U6, RNU6B, miR-16, and RNU-44 (37,68,76,82). However, the choice of housekeeping gene remains quite empirical because, to the best of our knowledge, there are no previous studies that have validated endogenous housekeeping control genes for miRNA normalization in LBC cervical samples.

In this study, we evaluated six candidate housekeeping genes for miRNA RT-qPCR data using LBC samples from patients who underwent cervical cancer screening. We analyzed the expression of five small nucleolar (sno) RNAs: RNU-44, RNU-47, RNU-48, RNU-49 and U6. The snoRNAs are a group of non-coding RNAs with variable length (80 to 1000 nt in yeast), mainly required for ribosomal RNA (rRNA) maturation (89). Many types of snoRNAs have been described in eukaryotes and each of them corresponds to a specific mode of transcription (90) and have been used as housekeeping genes for miRNA normalization (30,31). Some studies that have used miRNA profiling to discriminate cervical cancer from benign lesions selected RNU-44 and RNU-48 as endogenous controls for normalization of miRNA RT-qPCR data, mostly using tissue (26,32-34) and serum samples (35). However, our findings suggest that these snoRNAs are unsuitable for miRNA

Table II. Association between mean Cq values and histologic groups.

Housekeeping genes	HFS	CIN 1	CIN 2	CIN 3	CC	P-value
U6	27.76±0.48	26.47±1.38	22.44±12.71	24.40±1.77	25.41±1.97	0.060
miR-16	11.19±17.49	7.35±15.51	-	21.16±19.34	34.94±1.94	0.045[a]
RNU-44	30.03±3.49	28.78±2.06	31.25±2.41	26.74±1.47	20.60±11.60	0.004[a]
RNU-47	34.16±2.85	32.68±2.85	27.78±15.58	31.09±1.62	30.94±1.80	0.064
RNU-48	29.45±2.72	28.51±2.14	31.94±2.08	27.64±1.77	27.27±2.30	0.022[a]
RNU-49	30.67±2.73	29.77±2.07	23.84±13.34	28.51±1.03	28.08±0.95	0.128

-, no amplification. [a]P<0.05. CC, cervical cancer; CIN, cervical intraepithelial neoplasia; Cq, cycle quantification; HFS, healthy female subjects.

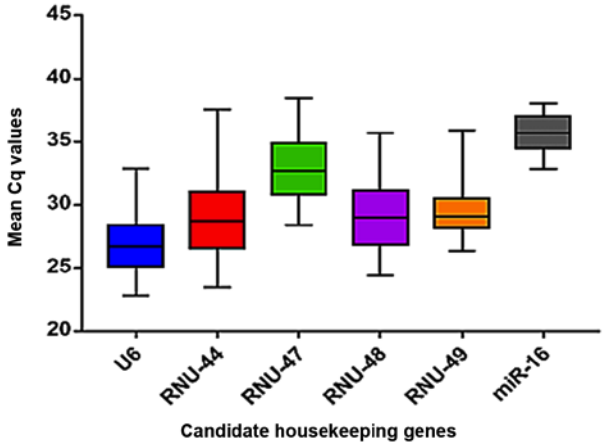

Figure 3. Box plots showing the distribution of mean Cq values of the six candidate housekeeping genes. Cq, cycle quantification.

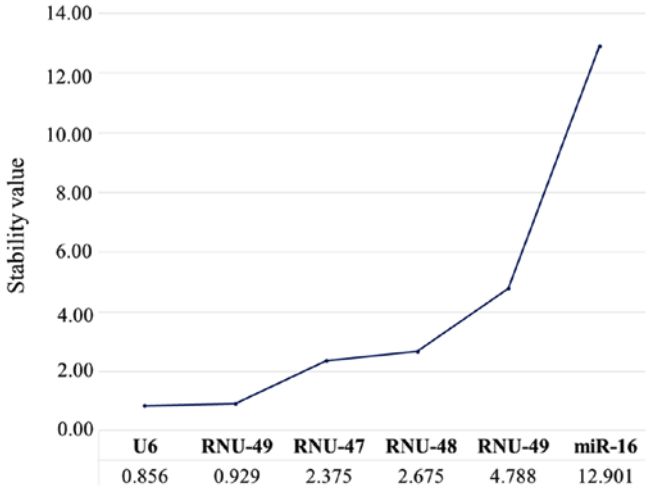

Figure 4. Stability values obtained using the NormFinder algorithm.

normalization in LBC samples, due to the higher number of amplification cycles required and the differential expression across distinct histologic groups. In addition, RNU-47 also required more amplification, confirming that it may not be an appropriate housekeeping gene. In contrast, RNU-49 and U6 could be amplified with fewer cycles and both have smaller variation according to NormFinder algorithm. Several studies have used U6 as housekeeping gene for RT-qPCR data normalization in cervical tissues (10-13), whole blood (62), serum (19,58,59) and air-dried Pap smears (71), but not previously in LBC samples. In agreement with other studies, our analyses indicate that U6 is the best housekeeping gene for LBC samples.

We also evaluated the miR-16 expression in LBC samples because it has been suggested as a housekeeping gene for cervical samples in other studies (25,45). There are doubts about the reliability of this gene for normalization because some studies have reported miR-16 as differentially expressed in CC. Zubillaga-Guerrero *et al* (91) demonstrated altered expression of miR-16 in CC, with miR-16 downregulating cyclin E1 (CCNE1) gene expression in cervical cancer cell lines. These data suggest a potential role of miR-16 in modulating cell cycle in CC and make it less likely to be a suitable control housekeeping gene. A recent systematic review also shown that miR-16 was deregulated and associated with cervical cancer progression (6). In our study, we confirmed that miR-16 was not a good endogenous control for RT-qPCR normalization in LBC samples because it presented higher variability expression across all samples-including amplification under background fluorescence in some cases-and altered expression in different histologic conditions.

In summary, our data demonstrates that U6 and RNU-49 are suitable housekeeping genes that can be used for miRNA RT-qPCR analyzes in LBC samples from patients who underwent cervical cancer screening. This is the first study that provide comprehensive information on the analytical performance of these genes for future normalizations of miRNA expression studies in LBC cervical samples that can be very useful for application of miRNAs in screening with LBC and had a clinical significance. However, future studies using RT-qPCR may to demonstrate in practice housekeepings U6 and RNU-49 may be excellent normalizers for liquid-based cytology (LBC) cervical samples. Thus, continued research efforts should be made about miRNA expression analysis wisely differentially expressed in these histological subtypes and LBC cervical samples using other tools for the stability analysis of housekeepings, such as geNorm.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RLC developed and led the overall study, conducted the data reviews and the analysis, and prepared the manuscript. DPP participated in setting up the PCR assay and prepared the manuscript. KCBS participated in setting up the PCR assay and critically revised the manuscript. AFE, RMVR and JHTGF designed and developed the study, and critically revised the manuscript. MMCMS conceived the study, provided advice during the study development and prepared the manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of the Barretos Cancer Hospital (approval no. 784/2014). Each research participant provided written informed consent for the publication of any data associated with the present study. All information that could be used to identify the study participants was kept confidential and encrypted in a secure database to ensure full confidentiality of clinical information, laboratory findings and the anonymity of each participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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