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Selection and validation of endogenous controls for microRNA expression studies in endometrioid endometrial cancer tissues

Anna Torres [a,c,*], Kamil Torres [a,b], Paulina Wdowiak [a], Tomasz Paszkowski [a,c], Ryszard Maciejewski [a]

[a] Laboratory of Biostructure, Chair and Department of Human Anatomy, Medical University of Lublin, Jaczewskiego 4, 20-090, Lublin, Poland
[b] General and Oncologic Surgery Department, Lublin County Specialist Hospital, 20-718, Al. Krasnicka 100, Lublin, Poland
[c] III Chair and Department of Gynecology, Medical University of Lublin, Jaczewskiego 8, Lublin, Poland

HIGHLIGHTS

- The study investigated expression of 12 candidate endogenous controls for microRNA qPCR studies in endometrioid endometrial cancer.
- RNU48/U75/NU44 were identified as stably expressed between malignant and normal tissues and could be used as reliable endogenous controls.
- The study presents an appropriate strategy for validation of candidate reference genes for any microRNA qPCR study.

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ABSTRACT

Objectives. microRNAs comprise a family of small, non-coding RNAs, which regulate gene expression at the posttranscriptional level. Multiple studies implicated important roles of microRNAs in various malignancies including endometrioid endometrial carcinoma (EEC). qPCR is widely used in the studies investigating microRNA expression. Relative quantification of microRNA expression requires proper normalization methods and endogenous controls are widely used for this purpose. The aim of this study was experimental identification of stable endogenous controls for normalization of microRNA qPCR expression studies in EEC.

Methods. Expression of twelve candidate endogenous controls (miR-16, miR-26b, miR-92a, RNU44, RNU48, U75, U54, U6, U49, RNU6B, RNU38B, U18A) was investigated in tissue samples obtained from 45 patients (30 EEC, 15 normal endometrium) using qPCR. Stability of candidate endogenous controls was evaluated using NormFinder, geNorm, BestKeeper and equivalency test. The results were then validated using larger group of samples.

Results. RNU48, U75 and RNU44 were identified as stably and equivalently expressed between malignant and normal tissues. Both NormFinder and geNorm indicated that those three snRNAs were optimal for qPCR data normalization in EEC tissues.

Conclusions. In conclusion, we suggest that average expression of those snoRNAs could be used as a reliable endogenous control in microRNA qPCR studies in endometrioid endometrial cancer. In addition to identifying suitable endogenous controls in EEC, our study presents an appropriate strategy for validation of candidate reference genes for any microRNA qPCR study.

Introduction

Endometrial cancer is the most common malignancy of the female reproductive tract and it is responsible for over eighty thousand new cancer cases in Europe each year [1]. The 5-year survival and prognosis is fairly good in patients diagnosed with early disease, whereas advanced stages are still connected with high mortality and survival rates less than 20% [2]. This is due to lack of screening methods for precancerous and early disease and not sufficient treatment modalities for advanced disease. Multiple molecular pathways have been investigated in endometrial cancer in the search for novel diagnostic, prognostic and treatment strategies [3]. New hypotheses on endometrial cancer pathogenesis emerged with the discovery of microRNAs (miRNAs) and their role in posttranscriptional gene expression regulation [4–6]. MiRNAs are implicated as important factors in carcinogenesis because they target and regulate expression of oncogens and tumor suppressor genes [6]. Recent studies reported altered expression of several miRNAs in endometrioid endometrial cancer (EEC). A large number of miRNAs were found up-regulated in EEC tissues with miR-200 family, miR-9, miR-203, miR-205, and miR-210 reported by at least two or

* Corresponding author at: Laboratory of Biostructure, Chair and Department of Human Anatomy, Medical University of Lublin, Jaczewskiego 4, 20-090, Lublin, Poland. Fax: +48 817423677.
E-mail address: anna.torres@wp.pl (A. Torres).

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more studies, whereas fewer miRNAs, including miR-410, miR-17-5p, miR-214, miR-99a,b, miR-199b, miR-100, miR-20a, miR-221, miR-222, and miR-424 were found down-regulated [7–18]. Moreover, miRNA tissue signatures have the potential of being used not only as diagnostic markers, but also as prognostic factors in EEC for they were found predictive of the recurrence, lymph node metastases and survival [17,18]. Furthermore, recently published studies suggest that miRNA plasma signatures could be used to distinguish patients with EEC from healthy controls [17–19]. Functional studies performed to date showed that single miRNAs (miR-145, miR-200 family, miR-204, miR-206) influenced proliferation, migration and survival of endometrial cancer cells [8,9,11,20–22]. Such observations are promising in regard to the development of novel diagnostic and therapeutic modalities. At the same time analysis of miRNA expression is difficult and many methodological issues are still not fully determined [23,24]. Therefore translational studies, which aim to discover new therapeutic or diagnostic approaches, should be based on the stringent and reliable methodology. One important issue in miRNA expression studies relates to proper normalization strategy, which is necessary to minimize systematic and technical bias introduced at each step of miRNA quantification process [25–27]. At present there is no consensus on the normalization strategy that should be used in miRNA qPCR studies. Three methods of miRNA normalization have been described in the literature: endogenous control-based, global mean expression and plate normalization factor [28–30]. The latter two methods are however suitable for large-scale studies. Therefore endogenous controls are still the method of choice for experiments focused on expression analysis of few miRNAs, which are often performed in translational studies.

Endogenous controls should be stably expressed and at the same time should undergo the same technical variation as target miRNAs. Although some authors suggested that certain miRNAs could be used as universal endogenous controls, the subsequent studies indicated that there was a possibility of their regulation in various diseases [28]. Therefore, similarly to mRNA expression analysis, a proper normalization of miRNA quantification requires a careful choice and validation of endogenous controls in the representative sample of the studied population [31].

Our search of miRNA profiling studies in endometrial cancer revealed that they relied on arbitrarily chosen endogenous controls. No previous report described an experimental identification and validation of suitable endogenous controls for normalization in endometrial cancer.

Thus we aimed to experimentally identify the most stable endogenous controls for normalization of miRNA qPCR expression studies in endometrioid endometrial cancer, which consists the most common histological type of the uterine malignancy. The candidate non-coding RNAs (ncRNAs) including nine snRNAs and snoRNAs and three miRNAs were chosen based on the previous studies, which suggested their stable expression across a range of tissues and cell lines [28,31–34]. In addition, none of the candidate non-coding RNAs chosen for analysis was regulated in any of the previously published endometrial cancer studies.

Materials and methods

Sample collection

Fresh tissue samples were obtained from 45 patients (30 EEC, 15 NE) and FFPE specimens from 58 patients (44 EEC, 14 NE). Preoperative diagnosis of EEC was subsequently confirmed by histological examination of the specimens obtained during surgery. FIGO staging was performed according to 2009 FIGO classification and EEC specimens were classified, according to the 2002 WHO classification in G1, G2 or G3. Study design was revised and granted approval from Medical University of Lublin Ethical Committee. Written informed consent was obtained from each study participant. Detailed characteristic of EEC patients was presented in Table 1.

All fresh tissue samples were collected during hysterectomies within 15 min after uterus excision. Normal endometrial samples were derived from patients operated due to pathologies other than of endometrial origin and comprised comparable numbers of proliferative and secretary phase endometrium. Immediately after sampling tissues were immersed and incubated in RNAlater (Ambion) for 24 h in 4 °C. After incubation tissues were stored in −80 °C until RNA extraction.

Candidate endogenous controls

The candidate endogenous controls (ECs) were chosen based on the literature suggesting their high abundance and stable expression across large panels of tissues and cell lines [28,31–34]. Characteristics of candidate ECs were presented in Table S5. The search of the literature confirmed that candidate non-coding RNAs included in our analysis were not regulated in endometrioid endometrial cancer tissues.

RNA isolation and analysis, reverse transcription and quantitative PCR

RNA isolation and analysis was performed using the mirVANA™ miRNA Isolation Kit (Ambion) RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE tissues (Ambion) for fresh and FFPE tissues respectively according to the manufacturer's protocol. RNA was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit and specific primers (Applied Biosystems). Single tube TaqMan® MicroRNA Assays and TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) were utilized for performing qPCR. Detailed description of the methods is provided in the Supplementary methods file (Supplementary file 2).

Data analysis

Raw qPCR data obtained from tissue samples were initially normalized with inter-plate calibrators and adjusted for reaction efficiency and were used as input in further stability analyses.

Stability of candidate ECs was evaluated using three different software applications, which are commonly utilized in the experimental validation of reference genes: NormFinder, geNorm and BestKeeper [35–37]. NormFinder is an ANOVA-based model, which returns standard deviation (SD) value, accumulated SD (Acc. SD) value and stability value, named variability. Analyses performed in NormFinder may consider groups and enable determination of intra-group and inter-group variations which make it possible to detect regulation of ECs between subgroups within the studied population [37,38]. This is the most important difference between NormFinder and other available

Table 1
Characteristics of endometrioid endometrial cancer patients.

Characteristic	Fresh RNAlater incubated tissues (n)	FFPE tissues (n)
FIGO stage		
IA	14	16
IB	9	8
II	–	5
IIIA	1	2
IIIB	3	–
IIIC1	3	5
IIIC2	–	6
IVA	–	1
IVB	–	1
Grade		
1	13	14
2	14	14
3	3	15
Myometrial invasion		
<0.5 of myometrial thickness	15	18
≥0.5 of myometrial thickness	15	26