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

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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

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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

algorithms [37]. The mathematical algorithms behind geNorm and BestKeeper are similar and identify best genes basing on pair-wise comparison between genes [35,36]. GeNorm calculates the stability values (M) for all candidate ECs using a step-wise pairwise variation excluding candidates with the lowest stability. The procedure is repeated until the two most stable ECs are predicted. Therefore geNorm sequentially eliminates non-optimal genes and returns the best pair of genes, characterized by stability value, named M value. The M value of 1.5 was defined as the upper limit for candidate ECs. Authors of geNorm suggested that values between 0.5 and 1.0 were characteristic for heterogeneous sample groups comprising healthy and cancer tissues [35]. geNorm also calculates the optimal number of genes suitable for normalization and returns the Vn/Vn + 1 value of such combination [35]. In the current study all candidate ECs included in the panel yielded M values below the threshold value of 1.5 and eight of them — below 1.0, which indicated their suitability for study design. BestKeeper determines gene stability basing on the standard deviations and coefficients of variance [36].

In the presented study we also investigated equivalent expression between EEC and normal endometrium groups using equivalence test and the fold change cut-off of 3 ($[-\varepsilon; \varepsilon] = [\log_2 1/3; \log_2 3] = [-1.585; 1.585]$) [39].

The stability of candidate ECs was assessed using three algorithms, which are commonly utilized in the experimental validation of reference genes: geNorm, NormFinder and BestKeeper. The mathematical algorithms behind geNorm and BestKeeper are similar [35,36]. GeNorm calculates the stability values (M) for all candidate ECs using a step-wise pairwise variation excluding candidates with the lowest stability. The procedure is repeated until the two most stable ECs are predicted. The M value of 1.5 was defined as the upper limit for candidate ECs. Authors of geNorm suggested that values between 0.5 and 1.0 were characteristic for heterogeneous sample groups comprising healthy and cancer tissues [35]. Different statistical approaches are applied in NormFinder, which is an ANOVA-based model. The most important difference between the two algorithms consists in possibility of considering the groups in NormFinder, which enables determination of intra-group and inter-group variations [37].

In the current study all candidate ECs included in the panel yielded M values below the threshold value of 1.5 and eight of them — below 1.0, which indicated their suitability for study design.

In order to perform relative quantification of miRNA expression in the validation part of the study mean Cq values of replicates (three RT and two qPCR replicates for each sample) were normalized with IPCs and adjusted for reaction efficiency and then normalized using four different normalization strategies: geometric mean of three experimentally chosen, stable ECs (RNU48, RNU44 and U75), miR-92a alone, geometric mean of RNU48/miR-92a and geometric mean (GM) of all amplified genes (RNU48, RNU44, U75 and 17 miRNAs), that were co-amplified as part of another study [17].

Normalized qPCR data were log transformed before statistical analysis. Distribution of data was estimated with D'Agostino–Pearson normality test. Fisher test was used to assess equality of variances. Student's *t*-test (with or without Welch correction) or Mann–Whitney test was used to compare between the groups as appropriate. Results are presented as mean with 95% confidence intervals (95% CI) or a fold change (FC) with 95% CI. Data analysis was performed using GenEx 5.3.4 (MultiD) and MedCalc (MedCalc Software) version 12.2.1.

All statistical tests were two-sided and $p < 0.05$ was considered to indicate statistically significant difference.

Results

Expression levels of candidate endogenous controls

All candidate ECs were expressed at suitable levels both in normal and in EEC tissues. In the case of RNU38B, RNU6B and U18A amplification was performed in 32 samples. Cq values of all candidate ECs were characterized in Supplementary Tables S1 and S2.

Comparison of raw Cq values revealed significant differences in the case of miR-16 ($p = 0.009$), RNU38B ($p = 0.018$) and U18A ($p < 0.0001$) (Fig. S1). Using the fold change cut-off of 3 ($[-\varepsilon; \varepsilon] = [\log_2 1/3; \log_2 3] = [-1.585; 1.585]$) the equivalent expression between normal and EEC groups was not confirmed for four candidate ECs: miR-16, miR-26b, miR-92a and U18A.

Analysis of candidate endogenous controls stability

NormFinder analysis

NormFinder analysis was initially conducted without considering the groups in the panels of 12 candidate genes (in the group of 32 samples) and 9 candidate genes (in the group of 45 samples) and the results are presented in Fig. 1. In both analyses RNU48 was identified as the most stable ncRNA followed by RNU44 and U75. Both analyses were also very concordant in terms of least stable ncRNAs: U49 and miR-26b.

Following, the EEC and NE groups were taken into account in order to inspect the inter-group variation, which is a differential expression counted based on average expression relative to the global mean of the genes in the panel. According to the assumption behind NormFinder algorithm, inclusion of potentially regulated candidate genes in the panel could interfere with the analysis. The threshold inter-group variation to disqualify genes from the panel depends on the difference between studied groups that is expected, however the expected regulation should usually be below 0.1 to 0.2 cycles. In the presented study we

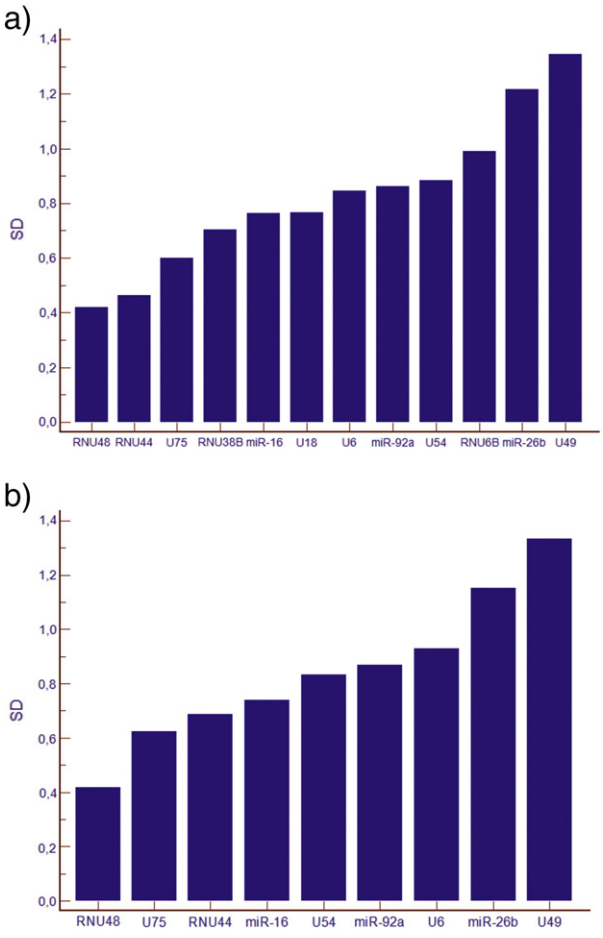

Fig. 1. NormFinder analyses performed without taking groups into account; (a) panel of 12 candidate genes (in the group of 32 samples); (b) panel of 9 candidate genes (in the group of 45 samples).

applied the threshold of 0.2 cycles for inter-group variation. Analysis of inter-group variation between EEC and NE groups revealed the highest variability values in the case of miR-92a, miR-26b and U18A (Table S3).

Inter-group variation analyses performed when groups were distinguished based on FIGO stage and histological grading confirmed those results (Table S4). Therefore miR-92a, miR-26b and U18A were excluded from the panel and analyses were repeated without considering the groups. The results obtained in both panels of candidate ECs were almost identical (Fig. 2). The highest decrease of Acc. SD was achieved for the three most stable ECs (RNU48, RNU44, U75) and addition of more ECs did not improve this result significantly (Fig. 2). The variability obtained for the best pair of ECs (RNU48/U75), when the groups were considered was as low as 0.076 (Table 2).

geNorm analysis

geNorm analysis identified RNU48, RNU44 and U75 as the most stable genes in two analyses performed in panels of 12 candidate genes (in the group of 32 samples) and 9 candidate genes (in the group of 45 samples). miR-92a was ranked as the fourth most stable reference gene in the geNorm analysis performed when the panel of nine genes was considered (Fig. 3a–b). Determination of number of genes required for optimal normalization performed by geNorm calculates the pairwise variation (Vn/Vn + 1) between sequential normalization factors. According to geNorm analysis performed in the panel of 12 genes normalization performed to average expression of four ncRNAs (RNU48, RNU44, RNU75, RNU38B) yielded the threshold value of 0.152. When the analysis was repeated in the panel including nine candidate ECs, five ncRNAs (RNU48, RNU44, U75, miR-92a, U54) yielded Vn/Vn + 1 value of 0.153 (Fig. 3c). However, this combination of ECs included miR-92a, which showed high inter-group variation in the NormFinder analysis.

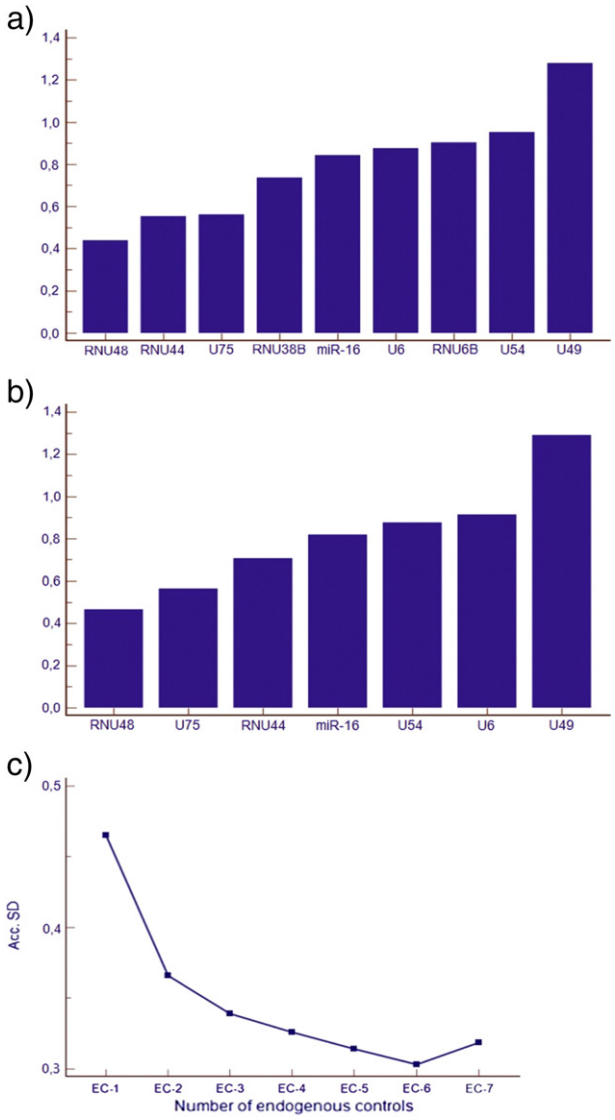

Fig. 2. NormFinder analyses performed without taking groups into account after excluding miR-92a, miR-26b and U18A; (a) panel of 9 candidate genes (in the group of 32 samples); (b) panel of 7 candidate genes (in the group of 45 samples); (c) accumulated SD obtained in NormFinder analysis after excluding miR-92a, miR-26b and U18A (in the group of 45 samples).

Table 2
geNorm M values and NormFinder variability after excluding miR-92a, miR-26b and U18A from the panel of candidate ECs.

Rank	NormFinder		geNorm	
	Candidate EC	ρ_{ig}	Candidate EC	M value
1	U75	0.098	RNU44	0.739
2	RNU48	0.101	RNU48	0.739
3	RNU44	0.131	U75	0.843
4	U6	0.181	miR-16	0.939
5	miR-16	0.191	U54	0.995
6	U54	0.193	U6	1.041
7	U49	0.295	U49	1.158
	Best combination of two ECs U75/RNU48 ρ_{ig} 0.076		Best two ECs RNU44, RNU48	

BestKeeper analysis

BestKeeper determines the stability of candidate reference genes basing on the coefficient of variation multiplied by 100. The lowest CqCV% value counted by BestKeeper was attributed to RNU38B, which was ranked fourth in the analyses performed by NormFinder and geNorm. However, the three most stable ECs ranked by NormFinder and geNorm were also characterized by fairly low CqCV% values of 3.63 (U75), 3.73 (RNU48) and 3.77 (RNU44). On the other hand ncRNAs, which were identified the least stable by NormFinder and geNorm presented the highest CqCV% values of 6.66 (miR-26b), 6.2 (U18A) and 4.86 (U49) (Table S1).

Validation of RNU48, RNU44 and U75 expression stability

In order to evaluate the expression stability of the three chosen ECs we compared their expression in the same group of patients, but with utilization of different fresh tissue samples for RNA isolation and different qPCR protocols. ECs were further evaluated in the new population of patients from which we obtained FFPE specimens. The results showed no difference in mean Cq values in the fresh tissue group, whereas in the FFPE group differences between Cq values were found for RNU48 and U75 (Figs. S2, S3).

Effect of reference genes on relative expression of target miRNAs

In order to evaluate the effect of candidate ECs on the target miRNA expression results, we assessed expression of five target miRNAs, which were previously reported up-regulated in EEC (miR-9, miR-141, miR-183, miR-200a, and miR-200c), using four different normalization strategies: geometric mean of RNU48/RNU44/U75, miR-92a alone, geometric mean of RNU48/miR-92a and geometric mean (GM) of all amplified genes.

The qPCR data used in this validation procedure was in part presented in our previous report [17] and was obtained basing on miRNA quantification performed in 104 tissue samples. The results are presented in Table 3.

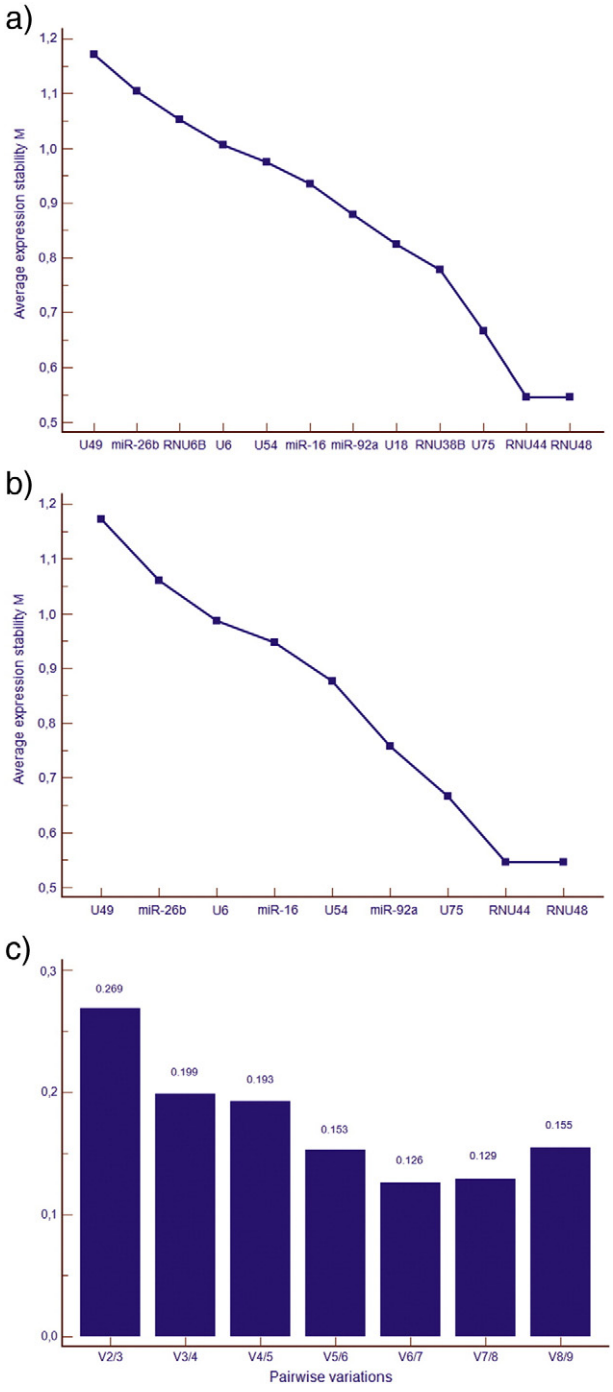

Fig. 3. geNorm analyses; (a) analysis performed in the panel of 12 candidate genes (in the group of 32 samples); (b) analysis performed in the panel of 9 candidate genes (in the group of 45 samples); (c) determination of the optimal number of ECs for normalization.

Discussion

Since their discovery miRNAs emerged as important molecules in cancer development, pathogenesis and progression [4]. They are also suggested as efficient markers in cancer diagnostics and outcome prediction [4]. Several studies are being conducted exploring treatment options associated with modulations of miRNA levels in vitro and in vivo. Any research conducted in miRNA field requires a reliable methodology, which enables accurate and biologically relevant quantitation of miRNA levels [25–27,31]. qPCR is one of the methods commonly used for this purpose. Similarly to mRNA expression analysis, miRNA quantitation with qPCR requires a proper normalization strategy to correct for non-biological variations [26,31]. As single miRNAs are thought to regulate hundreds of genes, proper normalization of miRNA studies may have a lot larger impact on biological relevance of acquired data in comparison to mRNA expression analysis. Although to date there is no consensus on miRNA qPCR data normalization, most experts agree that no single miRNA or small RNA can be used as a universal normalizer and the choice of optimal endogenous controls should be performed experimentally for each study design [31].

Involvement of miRNAs in the biology of EEC has been suggested in a number of studies, many of which applied qPCR and relative quantitation based on endogenous controls as a method of expression analysis. To the best of our knowledge none of those studies reported validation of the ECs used for normalization [7–15]. It is therefore the first report to describe a systematic and experimental assessment of candidate ECs, which could be used for normalization of miRNA qPCR data in EEC tissues. Due to the lack of data on miRNA stability in EEC, the panel of candidate ECs used in our study was chosen based on their high expression and stability in other normal and tumor tissues [28,31–34]. We also searched the EEC miRNA literature to make sure that none of the candidate miRNAs was previously reported regulated in EEC tissues [7–16].

Analyses performed by our group revealed that RNU48, RNU44 and U75 were the most stable ECs in EEC tissues. Both NormFinder and geNorm indicated that those three snRNAs were optimal for qPCR data normalization in EEC tissues. RNU48, RNU44 and U75 were also equivalently expressed between normal and cancer samples. RNU38B was highly ranked by BestKeeper and indicated as the fourth most stable reference gene in geNorm analysis. However, equivalency test did not confirm its equivalent expression between normal and cancer tissues. The algorithms were also very concordant in indication of least stable ECs, which further supported our results. It was suggested that a proper normalization strategy for relative gene quantification should include at least three to five ECs [40]. However, the choice of the number of genes used for normalization often depends on practical and economical rationales. NormFinder analysis performed in our study revealed that the highest decrease in variation was achieved with the three most stable ECs (RNU48, RNU44, U75). Addition of more ECs did not improve this result remarkably.

The three best ECs identified in the presented study belong to the class of small nucleolar RNAs (snoRNAs), approximately 70 nucleotides in length, which is responsible for site-specific chemical modification of target RNAs [41]. Small nucleolar and small nuclear RNAs were proposed as ECs for miRNA studies due to their similar size and high abundance in tissues [32]. A set of small RNAs was investigated in the large panel of tissues and cell lines and many of those molecules were found stably expressed [32]. However, some authors suggested that snRNA and snoRNAs are not optimal ECs for miRNA studies, as they may not reflect physicochemical properties of miRNA molecules [40]. Due to their larger size they may present different RNA isolation and reverse transcription efficiency as well as lower stability in comparison to miRNAs. Thus their utility was suggested less advisable, when the differences in overall abundance of miRNA could be anticipated, including experiments affecting miRNA biogenesis machinery or involving various tissues and cell lines in one analysis [24]. Nevertheless the most stable ECs chosen based on the analyses performed in this study belonged to small nucleolar RNA class. Although the candidate EC panel used in our study included three candidate miRNAs, which were previously found stably expressed in various tissues and cell lines, their expression did not appear stable in the studied set of EEC and NE samples.

In many studies the initial evaluation of candidate ECs was performed using a *t*-test performed on non-normalized Cq values, which in fact assume normalization to total RNA. In the case of miRNA studies such approach may be far more misleading than in mRNA expression profiling as miRNAs consist a very small fraction

Table 3
Effect of different normalization strategies on relative expression of target miRNAs.

miRNA	Normalization							
	RNU48, RNU48, U75		miR-92a		U48, miR-92a		GM	
	FC	p	FC	p	FC	p	F	p
miR-9	5.46	0.0002	−2.48	0.005	1.82	0.055	2.82	0.009
miR-92a	15.63	0.0002	–	–	–	–	7.37	<0.0001
miR-141	3.80	0.003	−3.2	0.001	1.16	0.64	1.94	0.011
miR-183	3.44	<0.0001	−4.22	<0.0001	1.18	0.54	1.75	0.028
miR-200a	3.61	0.0003	−3.61	0.0016	1.17	0.63	1.66	0.008
miR-200c	4.32	<0.0001	−2.89	0.00016	1.37	0.1	2.44	<0.0001

FC — fold change, GM — geometric mean of all amplified miRNAs/snoRNAs.

of total RNA and currently available measuring strategies do not enable reliable assessment of miRNA concentration in total RNA sample. Moreover such measurements can be strongly biased by RNA quality [25]. In our study ncRNAs characterized by high inter-group variation (NormFinder) were also differentially expressed based on *t*-test results, whereas in the case of RNU48, RNU44 and U75, no difference in expression was found in the *t*-test analyses performed in fresh tissues. However, when non-normalized Cq values of RNU48, RNU44 and U75 from EEC and control FFPE specimens were compared significant differences were found in expression of U75 and RNU48. As it was previously mentioned comparison of raw Cq values may not reflect true biological variation and in the case of FFPE specimens this result could be biased by RNA quality. Although it was proven that miRNAs are stable and resistible to chemical processing of FFPE specimens the recent study presented by Becker et al. indicated that total RNA quality adversely influenced results of miRNA profiling [25]. Such findings indicate that comparisons between groups comprised of fresh and FFPE samples could lead to erroneous results. Therefore, if the study involves both fresh and FFPE specimens the caution needs to be taken to include equal numbers of the two sample types in both normal and abnormal groups in order to minimize the risk of technical variation.

In addition to identifying stable ECs in EEC, our study revealed an important methodological issue, which should be concerned when choosing genes for the panel of candidate ECs. Inclusion of regulated genes in the panel analyzed with geNorm could lead to erroneous results, as this algorithm does not consider inter-group variation in gene expression. In our study geNorm ranked miR-92a the fourth most stable gene, whereas in NormFinder analysis miR-92a showed high inter-group variation. In order to evaluate such discordant results we decided to evaluate the differences in target miRNA (miR-9, miR-141, miR-183, miR-200a, and miR-200c) expression results after normalization to: geometric mean of RNU48, RNU44, and U75, miR-92a alone and geometric mean of RNU48 and miR-92a. In addition we have also performed normalization to the geometric mean (GM) of all amplified miRNAs and snoRNAs (20 molecules), as it was recently proposed that such normalization method could be efficiently used in smaller scale experiments [25]. To our surprise, the results obtained with miR-92a alone or a combination miR92a/RNU48 used as normalizers, showed significant differences compared to the results reached after normalization with the combination of RNU48, RNU44, RNU75 or GM. Interestingly, the latter two normalization strategies produced results, which were quite concordant.

To the best of our knowledge this is the first systematic approach to experimental identification of ECs for endometrioid endometrial cancer miRNA qPCR studies. In most of the previous studies various snRNAs, snoRNAs and rRNAs were used for normalization and authors did not report their experimental validation. Few studies were recently published discussing the subject of miRNA qPCR data normalization in other malignances and diseases. When compared to the results presented in those papers the stability values calculated for the best set of ECs in our study were similar or better. Davoren et al. examined stability of nine candidate ECs in breast cancer tissues and the pair of best reference genes yielded higher variability (0.221) and M (1.473) values in comparison to our results. Moreover, all of the EC combinations yielded V values above 0.15, as counted by geNorm [42]. The results obtained by Chang et al. in their colorectal cancer study revealed quite high M value of 0.994 for the best pair reference miRNAs, whereas the variability counted by NormFinder for the same pair was very low (0.003). The lowest V value in that study was 0.204 and was achieved with the normalization performed using five ECs [43]. Carlsson et al. found RNU24, RNU44 and RNU48 to be the most stable ECs in prostate cancer tissues [44]. NormFinder indices obtained by Shen et al. in uterine cervical tissues yielded variability of 0.176 for best single EC (miR-23a) [45]. In our study the variability of the best normalizer was very similar and yielded 0.1. In concordance with our results Shen et al. found a significant difference in miR-26a expression between normal and malignant samples. Further corresponding with our findings, U6 that is the most commonly used EC, was characterized by high M value in that study [45].

In conclusion, the results obtained using different statistical approaches were very concordant and indicated that RNU48, U75 and RNU44 were stably expressed in endometrioid endometrial cancer tissues and that combination of those snoRNAs could be used for normalization in miRNA qPCR studies in that malignancy. Moreover, we found that some of the small RNAs, which were previously used in EEC miRNA studies, should not be considered suitable for normalization, as they were not stably expressed in EEC tissues. It is generally stated that each qPCR study should begin with experimental validation of ECs. In addition to identifying suitable ECs in EEC, our study presents an appropriate strategy for validation of candidate reference genes for any miRNA qPCR study.

Conflict of interest statement

Authors state that there are no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygyno.2013.06.026.

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