Histol Histopathol (2017) 32: 941-950

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Micro-RNA signature of lymphovascular space involvement in type 1 endometrial cancer

Geoffroy Canlorbe[1,2,3], Mathieu Castela[1], Sofiane Bendifallah[2,4], Zhe Wang[1], Marine Lefevre[5], Nathalie Chabbert-Buffet[1,2], Selim Aractingi[1,6], Emile Daraï[1,2,3], Céline Méhats[7]* and Marcos Ballester[1,2,3]*

[1]INSERM, UMR S 938, University Pierre and Marie Curie, [2]Department of Obstetrics and Gynaecology, Tenon University Hospital, Assistance Publique des Hôpitaux de Paris (AP-HP), University Pierre and Marie Curie, [3]Institut Universitaire de Cancérologie (IUC), [4]INSERM UMR S 707, Epidemiology, Information Systems, Modeling, University Pierre and Marie Curie, [5]Faculté de médecine Paris 5 Descartes, [6]Department of Dermatology, Cochin University Hospital, Assistance Publique des Hôpitaux de Paris, University René Descartes and [7]INSERM U1016, CNRS 8104, Institut Cochin, University Paris Descartes, Paris, France

*These authors (CM and MB) contributed equally to this work

Summary. Objective. Lymphovascular space involvement (LVSI) is a major prognostic factor in type 1 endometrial cancer (EC). However, its use has been criticized because of poor subjectivity. MicroRNA signatures have recently been linked to EC pathologic characteristics. The aim of this study was to evaluate whether microRNA profiles of type 1 EC can be related to LVSI status and used as a tool to adapt therapy.

Study Design. MicroRNA expression was assessed by chip analysis and qRT-PCR in 12 formalin-fixed paraffin-embedded grade 2 EC specimens with positive LVSI and in 12 specimens with negative LVSI. Various statistical analyses, including enrichment analysis and a minimum p-value approach, were performed.

Results. The expression levels of microRNAs 34c-5p, -23b-5p, and 23c were significantly lower in the EC with positive LVSI compared to those with negative LVSI. Women with a microRNA-34c-5p fold change <0.15 were more likely to have positive LVSI status (92.3%) compared with those with a microRNA-34c-5p fold change >0.15 (0.0%), p<0.001. Furthermore, women with a microRNA-23b-5p fold change <0.51 were more likely to have positive LVSI status (90.0%) compared with those with a microRNA-23b-5p fold change >0.51 (21.4%), p=0.003.

Conclusion. This was the first study to investigate the relative expression of microRNA in type 1 EC according to LVSI status. This microRNA expression profile may provide a basis for further study of the microRNA function in EC, and be used as a diagnostic tool for LVSI status.

Key words: Endometrial cancer, Lymphovascular space involvement, MicroRNA, MicroRNA-34c, MicroRNA-23

Offprint requests to: Dr. Geoffroy Canlorbe, MD, Service de Gynécologie-Obstétrique, Hopital Tenon, 4 rue de la Chine, 75020 Paris, France. e-mail: geoffroy.canlorbe@aphp.fr

DOI: 10.14670/HH-11-859

Introduction

Endometrial cancer (EC) is classified into two main clinicopathologic types: type 1, the much more common endometrioid adenocarcinoma (80-90%); and type 2 which comprises non-endometrioid subtypes such as serous, clear cell and undifferentiated carcinomas, as well as carcinosarcoma (10-20%) (Morice et al., 2015; Colombo et al., 2016). Currently, the surgical management of type 1 ECs depends on prognostic factors such as histologic grade, myometrial involvement, and lymphovascular space involvement (LVSI) status.

Among these factors, LVSI is considered as a major determinant of recurrence and overall survival (Bendifallah et al., 2014). The European Society of Medical Oncology (ESMO) / European Society of Gynaecological Oncology (ESGO) / European Society for Radiotherapy & Oncology (ESTRO) joint committee recently recognized the adverse prognostic role of LVSI in type 1 grade 2 EC by defining a new subdivision of the intermediate risk group for recurrence to guide therapy: stage I, type 1, grade 2 ECs with positive LVSI status are now classified as being at high or intermediate

risk for recurrence, regardless of depth of myometrial invasion (Colombo et al., 2016). However, the use of LVSI as a prognostic criterion has been criticized for its subjectivity, poor reproducibility (Song et al., 2012) and is responsible for discrepancies between preoperative and actual risk-group determination based on final histology. These discrepancies are a major source of inaccurate or inadequate initial surgical staging exposing women to the risk of additional surgery or unnecessary adjuvant therapies. Thus, additional tools are needed to better assess LVSI status and thereby identify women with true high-risk disease.

MicroRNAs (miRNAs) are noncoding, ~22 nucleotide-long RNAs that regulate gene expression at the posttranscriptional level (Guo et al., 2010). Recently, miRNA signatures have been linked to the pathologic characteristics and prognosis of EC (Canlorbe et al., 2016). However, there are no data about LVSI status-associated miRNA regulation in EC.

The aim of this study was to evaluate whether miRNA profiles of presumed early-stage type 1 grade 2 endometrioid adenocarcinomas can be related to LVSI status and used as a tool to adapt surgical staging and adjuvant therapy.

Materials and methods

Many of the methods related to our patient cohort, miRNA extraction, microarray hybridization and analysis, qRT-PCR, and minimal p-value approach have been previously published (Canlorbe et al., 2016).

Experimental design

Approval for the present study was obtained from the local Medical Ethics Committee (CPP Ile-de-France V; e-4-15) and all the women had given informed written consent allowing their data or tissue to be used for medical research purposes.

The experimental design for profiling changes in miRNA according to LVSI status in endometrioid grade 2 formalin-fixed and paraffin-embedded (FFPE) primary EC tumor specimens is shown in Fig. 1. Twenty-four women with presumed early-stage EC on preoperative imagery (i.e. women with a primary tumor confined to the corpus uteri) who underwent primary surgical treatment (including total hysterectomy, bilateral salpingo-oophorectomy and systematic nodal staging) between January 2003 and December 2012 in Tenon University Hospital-APHP were enrolled in the study. Twelve of these women had positive LVSI status (positive LVSI) on final analysis and met the following inclusion criteria: endometrioid adenocarcinoma, grade 2, and FIGO (International Federation of Gynecology and Obstetrics) stage I (regardless of nodal status according to final analysis). The remaining 12 women, with negative LVSI status (negative LVSI), met the same inclusion criteria and were used as control subjects. One-to-one matching was performed according to nodal involvement criteria. The exclusion criteria were as follows: previous malignancies, history of chemotherapy or radiotherapy, inflammatory disease, or Lynch syndrome.

The clinical and pathologic variables of the women were extracted from maintained EC databases. Epidemiologic and histologic characterizations between women with negative or positive LVSI status are represented in Table 1.

Histologic characteristics

All postoperative pathologic analyses were performed by a dedicated gynecologic oncology pathologist and reviewed blind by a second pathologist to confirm the presence/absence of LVSI. Histologic grade 2 is defined by 6% to 50% of solid nonsquamous, nonmorular pattern. The presence of grade 3 nuclei involving more than 50% of the tumor increases the grade by one (Kurman, 2014). Therefore, histologic grade 1 defined by 5% or less of a solid nonsquamous, nonmorular growth pattern but with more than 50% of grade 3 nuclei is considered grade 2 EC. A tumor is considered LVSI positive when tumor emboli are found within a space clearly lined by endothelial cells on hematoxylin and eosin (H&E)-stained sections (Briët et al., 2005).

RNA extraction from FFPE tissues, microarray hybridization (GEO: GSE75968) and data analysis

miRNA analysis according to LVSI status was done in post-operative specimen. FFPE tissues were obtained from the hysterectomy specimens. Briefly, as previously reported, 10 μm sections of pathologist confirmed regions of invasive carcinoma were microdissected. Total RNA was extracted using the miRNeasy FFPE Kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions.

Microarray analysis was conducted on 12 distinct samples: six samples with positive LVSI (three individual specimens with nodal involvement and three pools of three different specimens without nodal involvement) and six samples with negative LVSI (three individual specimens with nodal involvement and three pools of three different specimens without nodal involvement). Microarray hybridization on miRNA 4.0 chips (Affymetrix) was conducted at the genomic platform of the Institut Cochin, Paris. Specific miRNA analysis was performed using Partek® Flow® software, version 3.0 (Partek Inc., St. Louis, MO, USA). CEL files were imported and normalized using Robust Multi-array Averaging (RMA) (Irizarry et al., 2003).

Genes with a nominal p-value≤0.05 were considered to be differentially expressed. Among these, genes showing a variation of 1.5 were retained for further analysis.

To estimate the biologic effects of the differentially expressed miRNAs, lists of 3050 validated target genes

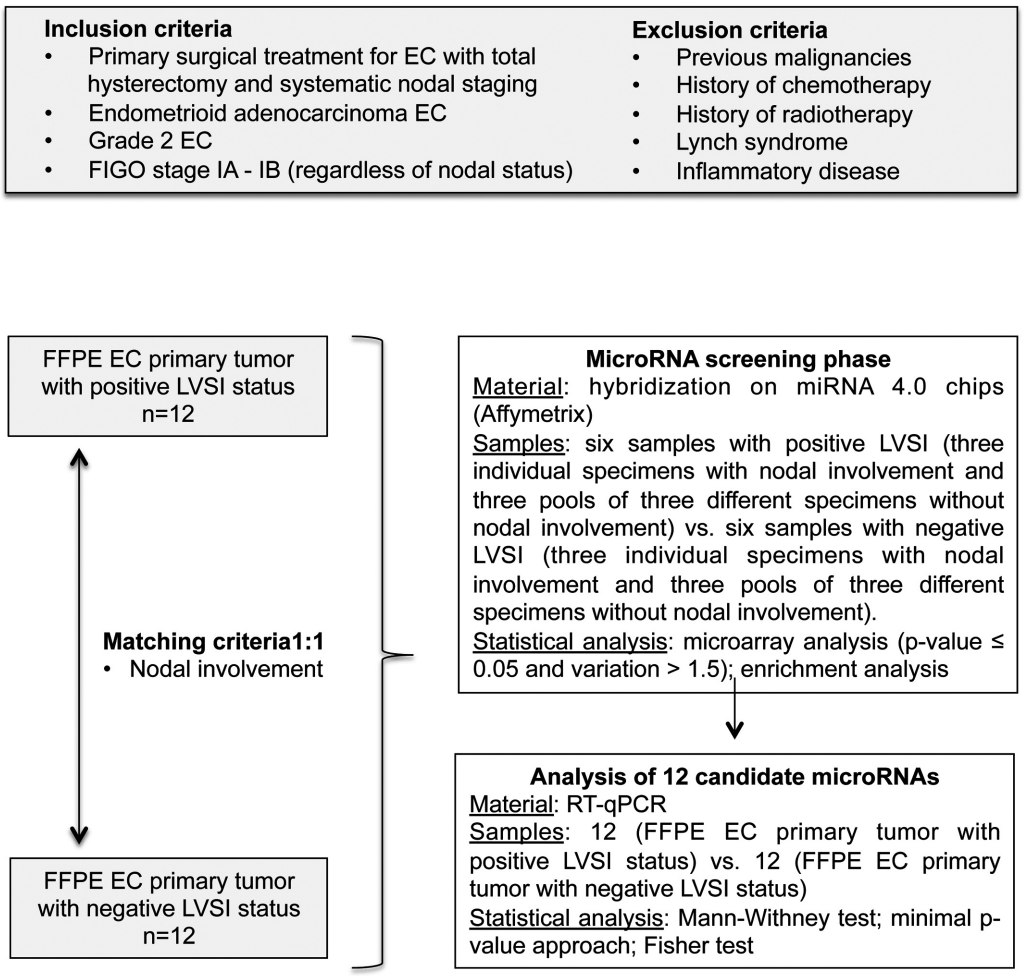

Fig. 1. Flowchart describing the constitution of groups and the experimental design. EC, endometrial cancer; FFPE: formalin-fixed paraffin-embedded; FIGO, International Federation of Gynecology and Obstetrics; LVSI, lymphovascular space involvement; RT, reverse transcription; qPCR, quantitative real time polymerase chain reaction.

Table 1. Epidemiologic and histologic characterizations between women with negative or positive LVSI status.

	EC FFPE primary tumor Negative LVSI status n=12	EC FFPE primary tumor Positive LVSI status n=12	P-value
Age (years), median (IQR)	69 (64-72)	68 (66-76)	0.85
Hypertension, n (%)	4 (33.3%)	4 (33.3%)	1
Diabetes n (%)	2 (16.7%)	0 (0%)	0.48
Parity, median (IQR)	2 (1-3)	1 (0-2)	0.13
BMI, median (IQR)	25.5 (20.75-30)	22.5 (22-28.75)	0.97
<30, n (%)	7 (70 %)	8 (80 %)	1
≥30, n (%)	3 (30 %)	2 (20 %)	
FIGO Stage, n (%)			
IA	8 (66.7%)	5 (41.7%)	0.41
IB	4 (33.3%)	7 (58.3%)	
Histologic grade, n (%)			
Grade 2	12 (100%)	12 (100%)	1
Tumor size (mm), median (IQR)	40 (34-46)	35 (25-50)	0.36
Nodal involvement, n(%)			
Yes	3 (25%)	3 (25%)	1
No	9 (75%)	9 (75%)	
Time between surgery and sample analysis (months), median (IQR)	30 (10-53)	27 (15-92)	0.30

EC, endometrial cancer; FFPE, formalin-fixed paraffin-embedded; LVSI, lymphovascular space involvement; IQR, interquartile range; BMI, body mass index; FIGO, International Federation of Gynaecology and Obstetrics.

were determined using currently available databases, including Tarbase® and Mirtarbase®. Gene Ontology (GO) enrichment analysis was performed on the lists using Genomatix GePS (release 2.4.0, Genomatix BH, Munich).

qRT-PCR validation

RT reactions were performed with the miScript®II RT Kit, using 1 μg of total RNA, according to the manufacturer's instructions (Qiagen, Courtaboeuf, France). miRNA expression was analyzed by real-time PCR using miScript SYBR® Green PCR Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. miRNA primers were from Qiagen (Courtaboeuf, France). Relative expression was calculated using the comparative Ct method (2-ΔΔCt). Both SNORD61 and RNU6 were used as endogenous controls for data normalization.

For the results from the qRT–PCR on miRNA expression data are expressed as means ± SEM. Means between two groups were compared using the Mann Whitney test. $p<0.05$ was considered to be statistically significant. GraphPad Prism version 5 was used for analysis of tissue samples (GraphPad Software, La Jolla, CA, USA).

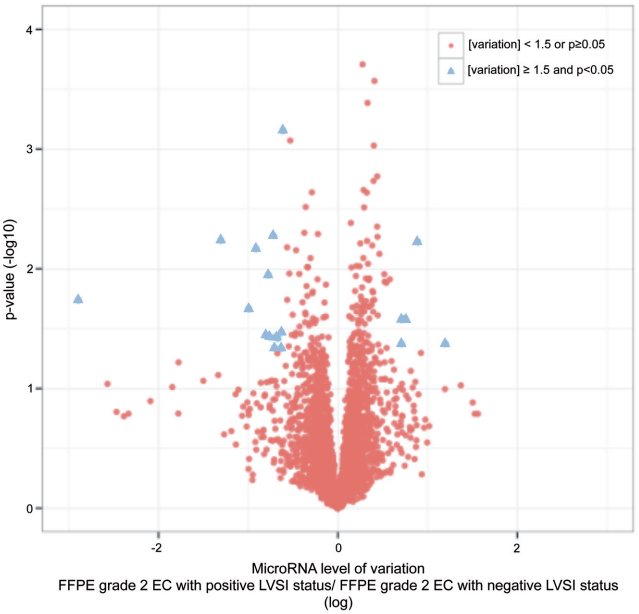

Fig. 2. Volcano plot. Abscissa is the logarithmic value of the level of variation (LogRatio) and ordinate is the negative logarithm of the statistical value (-log (p)) of fluorescence intensities of the hybridized probes from positive or negative LVSI status samples. There was more than a 1.5-fold change in normalized fluorescence intensity of 18 microRNAs (blue triangle) between the positive LVSI status vs. negative LVSI status groups ($p<0.05$): 13 decreased in intensity (on the left) and 5 increased in intensity (on the right). EC, endometrial cancer; FFPE, formalin-fixed paraffin-embedded; LVSI, lymphovascular space involvement.

Table 2. List of the downregulated (fold-change <-1.5, p-value <0.05) and upregulated (fold-change >1.5, p-value <0.05) microRNA between positive LVSI status vs. negative LVSI status FFPE early stage grade 2 EC primary tumor specimen.

Name	Fold Change	P-Value
Downregulated microRNAs		
miR-34c-5p	-7.44	0.018
miR-6511a-3p	-2.47	0.006
miR-335-5p	-1.99	0.022
miR-6080	-1.88	0.007
miR-23b-5p	-1.74	0.039
miR-23c	-1.71	0.011
miR-4274	-1.65	0.005
miR-660-5p	-1.63	0.046
miR-3187-5p	-1.61	0.037
miR-3131	-1.59	0.038
miR-5189-5p	-1.55	0.046
miR-502-5p	-1.55	0.034
miR-4731-5p	-1.53	0.0007
Upregulated microRNAs		
miR-575	2.30	0.042
miR-7975	1.86	0.006
miR-6760-5p	1.68	0.026
miR-5681a	1.64	0.027
miR-7-1-3p	1.64	0.042

LVSI, lymphovascular space involvement; EC, endometrial cancer; FFPE, formalin-fixed paraffin-embedded.

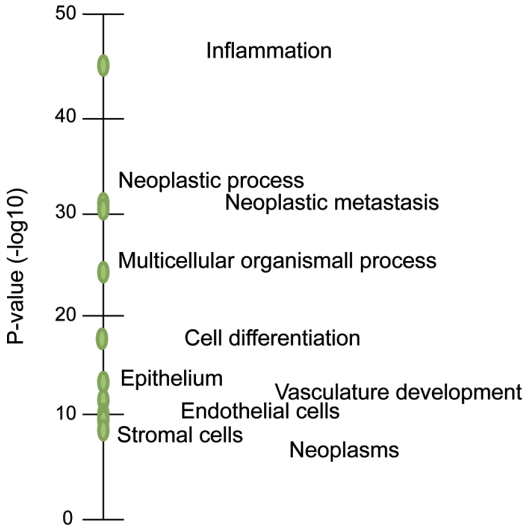

Fig. 3. Enrichment analysis. Gene ontology enrichment analysis was performed (Genomatix GePS (release 2.4.0, Genomatix BH, Munich)) using the list of all known validated targets for the miRNAs exhibiting at least a 1.5-fold change, with significant value ($p<0.05$), in the early stage grade 2 EC primary tumors with positive LVSI status.

Optimal miRNA fold-change cut-offs correlated with LVSI status

For qualitative analysis, we calculated optimal cut-offs for each miRNA to correlate semi-quantitative expression and LVSI status. The optimal fold-change cut-off was determined by a minimum p-value approach.

Statistical analysis

Unless otherwise specified, data were managed with an Excel database and analysed using R 3.1.3 software, available online.

Results

Distinct miRNA signatures of grade 2 EC primary tumors with positive LVSI status

We focused our study on the 2,560 probes containing sequences for mature miRNAs. Among them, 93 had consistent normalized fluorescent intensities between the two groups (positive LVSI vs. negative LVSI) ($p<0.05$). As illustrated by the volcano plot representation (Fig. 2), there was more than a 1.5-fold change significant difference in the normalized fluorescence intensity of 18 of these miRNAs between the positive and the negative LVSI groups: 13 miRNAs (miR-34c-5p, miR-6511a-3p, miR-335-5p, miR-6080, miR-23b-5p, miR-23c, miR-4274, miR-660-5p, miR-3187-5p, miR-3131, miR-5189-5p, miR-502-5p, and miR-4731-5p) had a decreased expression and five miRNAs (miR-575, miR-7975, miR-6760-5p, miR-5681a, and miR-7-1-3p) had an increased expression in samples from the positive LVSI group compared to samples from the negative group (Table 2).

GO term enrichment analysis was then performed using the list of all known validated targets for the miRNAs exhibiting at least a 1.5-fold change, with significant value ($p<0.05$), in the EC primary tumors with positive LVSI. Genes involved in the neoplastic process, neoplastic metastasis, vasculature development, and neoplasms were specifically enriched (Fig. 3).

Evaluation of miRNA expression by real time qRT PCR analysis

A qRT PCR assay was used to confirm the expression of the miRNAs that had been selected from the previous step. The expression levels of three miRNAs were significantly lower (miR-34c-5p, miR-23b-5p, and miR-23c; $p<0.0001$, 0.006, and 0.046, respectively) in the EC primary tumors with positive LVSI compared to those with negative LVSI. miR-660 was downregulated, with no significant difference ($p=0.08$), in the EC primary tumors with positive LVSI compared to those with negative LVSI (Fig. 4).

Correlation between miRNA expression and LVSI status grade 2 EC

Optimal cut-offs denoting the strongest correlation between quantitative expression of the miRNAs that had been selected from the previous step and LVSI status are summarized in Fig. 5. The fold-change cut-offs defined were 0.10, 0.51, 1.1, and 1.2 for miR-34c-5p, miR-23b-5p, miR-23c, and miR-660, respectively. miR-34c-5p and miR-23b-5p had the most significant p-values: <0.001 and 0.003, respectively. We compared LVSI status according to the cut-offs previously determined: women with grade 2 EC and a miRNA-34c-5p fold-change <0.15 were more likely to have positive LVSI status (n=12; 92.3%) compared with those with an miRNA-34c-5p fold-change >0.15 (n=0; 0.0%), $p<0.001$; women with grade 2 EC and an miRNA-23b-5p fold-change<0.51 were more likely to have positive LVSI status (n=9; 90.0%) compared with those with a miRNA 23b-5p fold-change >0.51 (n=3; 21.4%), $p=0.003$ (Table 3).

Discussion

Our results show that in endometrioid grade 2 EC, women with positive LVSI status have different miRNA profiles from those with negative LVSI status. Moreover, we found that final LVSI status can be accurately assessed using miRNA expression levels.

Table 3. Optimal fold-change cut-offs denoting the strongest correlation between micro RNA expression and LVSI status in early stage grade 2 EC FFPE primary tumor specimens.

		EC FFPE primary tumor Negative LVSI status N=12	EC FFPE primary tumor Positive LVSI status N=12	p-value
miR-34c-5p	FC<0.15	1 (7.7 %)	12 (92.3 %)	<0.001
	FC>0.15	11 (100 %)	0 (0 %)	
miR-23b-5p	FC<0.51	1 (10 %)	9 (90 %)	0.003
	FC>0.51	11 (78.6 %)	3 (21.4 %)	
miR-23c	FC<1.1	7 (36.8 %)	12 (63.2%)	0.037
	FC>1.1	5 (100 %)	0 (0 %)	
miR-660	FC<1.2	6 (33.3%)	12 (66.7%)	0.014
	FC>1.2	6 (100 %)	0 (0 %)	

EC, endometrial cancer; FFPE, formalin-fixed paraffin-embedded; LVSI, lymphovascular space involvement; FC, fold-change.

The crucial issue in managing patients with EC is to determine the risk group of recurrence on preoperative biopsy and MRI findings to adapt the surgical strategy. Previous studies have shown that discrepancies existing between pre- and postoperative grade mainly for grade 1 EC and that LVSI status is very rarely assessed on biopsy (Frumovitz et al., 2004). This is of major importance as the presence of LVSI is considered the first step in metastatic spread and is a predictor of nodal involvement, recurrence risk, and overall survival (Briët et al., 2005; Guntupalli et al., 2012), especially for early-stage type 1 EC (O'Brien et al., 2009). According to our chip analysis, 13 miRNAs were downregulated and five upregulated more than 1.5-fold between the positive and the negative LVSI groups. Also, according to the enrichment analysis, the validated target genes for these differentially expressed miRNAs were almost exclusively involved in carcinogenic pathways, underlining the strength of our analysis. Our results are in agreement with those of previous studies showing that miRNAs can act as tumor suppressors or oncogenes in various cancers (Farazi et al., 2013) including EC (Kontomanolis and Koukourakis, 2015; Sianou et al., 2015). Furthermore, recent evidence indicates that several miRNAs, including miR-34, regulate the metastatic process through the expression of epithelial to mesenchymal transition (EMT) - transcription factors (TF)s or EMT-activating signalling pathways (SNAIL1/SNAIL2, basic helix-loop-helix (bHLH), E47, E2-2, TWIST1/TWIST2, and ZEB (ZEB1/ZEB2) families) (Díaz-López et al., 2014) that act as E-cadherin repressors (Peinado et al., 2007) and, ultimately, enhance cell migration and invasiveness in various cancers including EC (Montserrat et al., 2012).

After validation by qRT-PCR of the miRNAs, we found that miR-34c-5p, miR-23b-5p, and miR-23c emerged as being particularly relevant in determining LVSI status. The miR-34 family has been found to play a key role as a tumor suppressor (Agostini and Knight, 2014). It is a direct target of the tumor suppressor gene p53 and acts on apoptosis and cell cycle through the repression of many proteins involved in the regulation of these two biologic processes. This is totally in agreement with the recent molecular classification of EC which

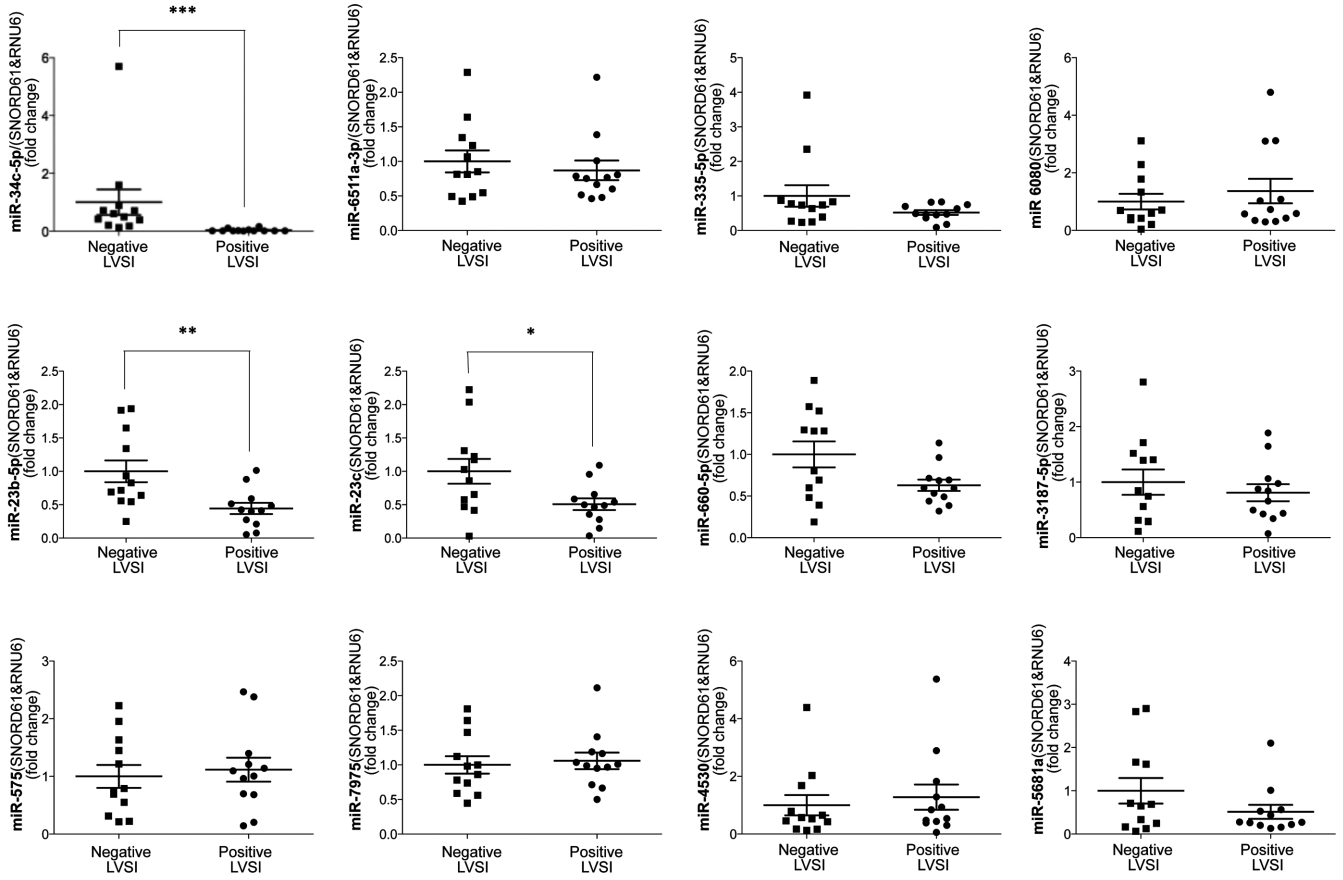

Fig. 4. qRT-PCR assay. The expression levels of 3 microRNAs (miR-34c-5P, miR-23b-5p, and miR-34c) were significantly lower in the early stage grade 2 EC FFPE primary tumor samples with positive LVSI status compared to those with negative LVSI status. Mann-Whitney test, *p<0.05; **p<0.01; ***p<0.001; mean ± SEM. EC, endometrial cancer; FFPE, formalin-fixed paraffin-embedded; LVSI, lymphovascular space involvement.

considers the p53 mutation to be an accurate prognostic marker in EC (Morice et al., 2015). In particular, the miR-34 family members bind to the 3'-UTRs of genes such as CDK4 and CDK6 (cell cycle proteins), Bcl-2 (an apoptosis regulator), SNAIL (epithelial mesenchymal transition) and CD44 (migration and metastasis), thus repressing their expression (Agostini and Knight, 2014). miR-34c regulates the blood tumor barrier permeability via myc-associated zinc-finger protein (MAZ)-mediated expression changes of zonula-occluden-1 (ZO-1), occludin, and claudin-5 (Zhao et al., 2015a,b). Yet little research has been conducted in EC, apart from a recent study by Canlorbe et al. (2016) who showed that the downregulation of miR 34c-5p was associated with nodal involvement in early-stage grade1-2 EC. Furthermore, a functional study by Li et al. demonstrated that miR-34c acts as a tumor suppressor in HEC-1-B cells (Li et al., 2015).

According to a recent review by Donadelli et al., miR-23b is dysregulated in tumors compared with normal tissues (Donadelli et al., 2014). Furthermore, the authors report that miR-23b is comprised in the miRNA signature of various tumors including endometrial carcinosarcoma (Castilla et al., 2011), uterine sarcomas and mixed epithelial-mesenchymal uterine tumors (Kowalewska et al., 2013). Pellegrino et al. demonstrated that miR-23b was involved in cytoskeletal remodelling through the enhancement of cell–cell interactions, reduction of cell motility and invasion during cancer progression (Pellegrino et al., 2013). Furthermore, miR-23b has been known to act as a suppressor of EMT by targeting HMGA2 (Liu et al., 2016), snai1 and zeb1 transcription factors (Campos-Viguri et al., 2015), ATG12 (Wang et al., 2013), Zeb-1 (Majid et al., 2013) and Akt (Majid et al., 2012). Once again, there has been little research conducted in EC, apart from a study by Castilla et al. who analyzed the miRNA signatures associated with EMT in human carcinosarcomas (Castilla et al., 2011). They demonstrated that a loss of epithelial characteristics, including cadherin switching and the acquisition of a mesenchymal phenotype, was accompanied by changes in the miRNA expression profile and the upregulation of all the E-cadherin repressors analyzed. In particular, miR-23b was downregulated in the mesenchymal part of the endometrial carcinosarcomas (Castilla et al., 2011). Finally, the expression of miR-23b has been studied in cervical cancer cell lines by Campos-Viguri et al. who

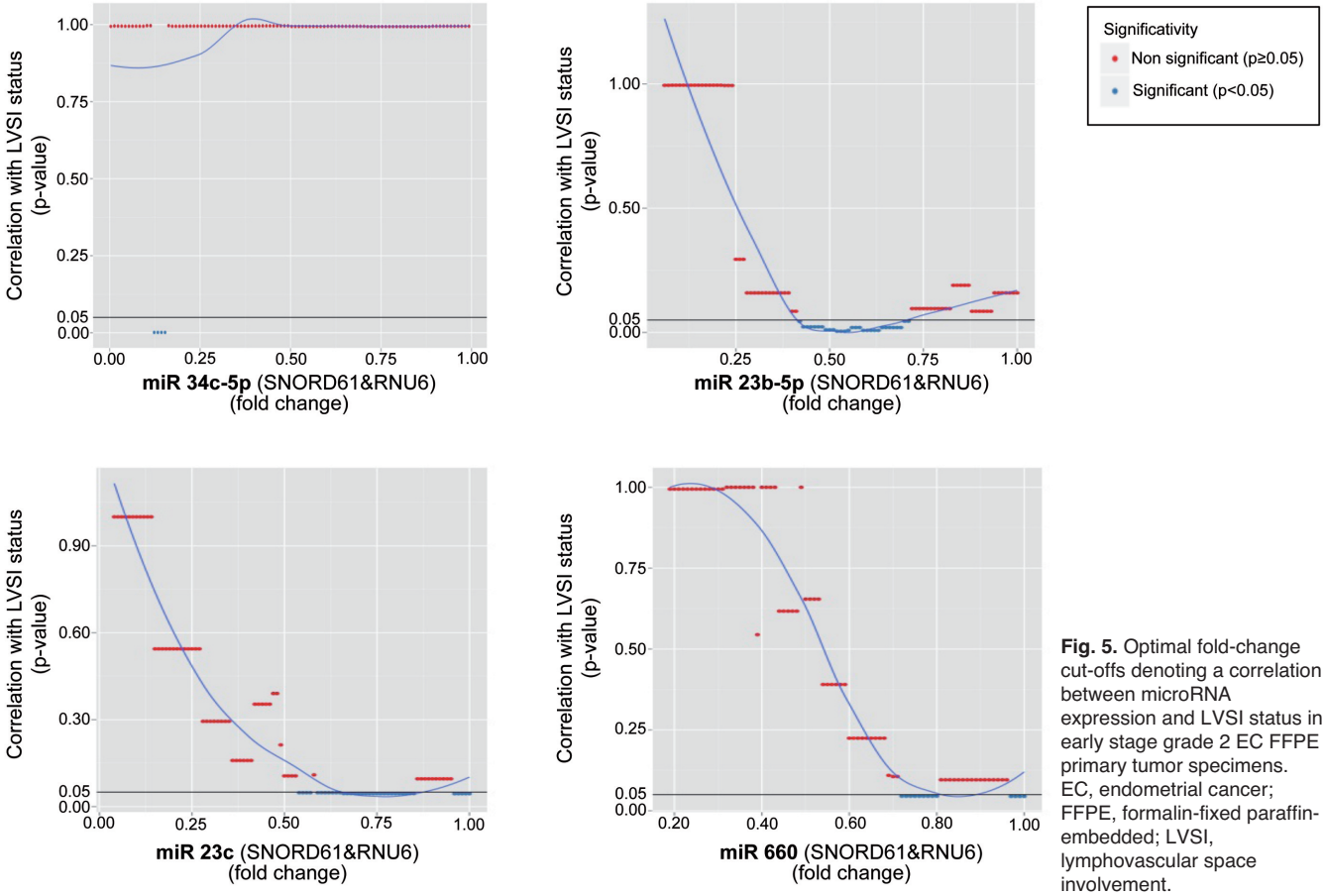

Fig. 5. Optimal fold-change cut-offs denoting a correlation between microRNA expression and LVSI status in early stage grade 2 EC FFPE primary tumor specimens. EC, endometrial cancer; FFPE, formalin-fixed paraffin-embedded; LVSI, lymphovascular space involvement.

reported that this microRNA may influence the expression of uPa, c-Met and Zeb1 (Campos-Viguri et al., 2015).

Pathologic tools have been developed to evaluate LVSI status in EC. Jiang et al. analyzed the expression of the oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) in 186 women with endometrioid EC and reported that the triple negative phenotype was associated with positive LVSI (Jiang et al., 2012). Moreover, Tomica et al. showed that a loss of both ER and PR in EC cell nuclei was correlated with positive LVSI (Tomica et al., 2014). Tumor Necrosis Factor alpha-Induced Protein 8 (TNFAIP8) (Liu et al., 2014), hyaluronic acid binding protein 1 (HABP1) (Zhao et al., 2015a,b), HAAA+ (ATPases associated with various cellular activities) nuclear coregulator cancer-associated (ANCCA) protein expression in EC has been found to be correlated to positive LVSI (p=0.011). Furthermore, ploidy, S-phase fraction (Song et al., 2012), p53 expression (Osmanağaoğlu et al., 2005), CRP 1846C>T genetic polymorphism (Kito et al., 2015) correlate with positive LVSI status. Recently, an increased infiltration of CD163(+) tumour-associated macrophages (Kübler et al., 2014) and the CD8 and CD4 cell counts, regulatory T cell (Treg) count, and Treg/CD8 and Treg/CD4 ratios were significantly higher in EC patients with positive LVSI than in those with negative LVSI (Yamagami, 2011). Despite potential relevance, all these analyses were performed on uterine specimens and need to be validated on preoperative samples. An answer could be provided by the ongoing PIPENDO (PIpelle Prospective ENDOmetrial carcinoma) study which aims to define the optimal panel of prognostic biomarkers in EC (Visser et al., 2015). Despite a potential relevance, all these analyses were performed on uterine specimens and need to be validated on preoperative samples. An alternative assessment of LVSI status could be provided preoperatively by transvaginal colour Doppler ultrasound (Alcázar et al., 2002), 18F-FDG PET/CT (Kitajima et al., 2015) or MRI. Indeed, various studies have demonstrated that peritumoral enhancement (Fujii et al., 2015), SUVmax (Lee et al., 2011), a tumor volume ratio greater than or equal to 25% (Nougaret et al., 2015) are correlated with LVSI. However, an external validation of these data is required due to the risk of intra- and inter-observer variability. Furthermore, most of these results are from individual analysis and further studies are needed to confirm their clinical value.

Some limitations of the study should be underlined. First, the retrospective nature of the study cannot exclude all bias. Second, myometrial involvement, an independent predictor of LVSI status, was not strictly identical between LVSl-positive and negative grade 2 EC groups. Also, we cannot distinguish cases with superficial and deep myometrial invasion that may lead to an analysis bias. Third, to provide a diagnostic tool, this analysis should be validated on preoperative samples. Unfortunately, assessing miRNA expression in preoperative biopsy samples might be difficult due to the contamination of normal endometrial or myometrial tissue. A heterogenic sample, with distinct miRNA profiles could lead to a wrong analysis. Two recent studies have demonstrated that circulating miRNAs have a high accuracy in diagnosing endometrioid EC (Torres et al., 2013; Wang et al., 2014). The miRNA described in our study may be preoperatively assessed in blood samples of women with type 1 grade 2 EC and might serve as a novel, non-invasive biomarker in the future. Further study should validate on novel, large series of patients the diagnosis performance of candidate miRNAs proposed.

Conclusion

In conclusion, this is the first study investigating the relative expression of mature miRNA genes in type 1 grade 2 EC primary tumors according to the LVSI status. This miRNA expression profile may provide a basis for further study of the miRNA function in endometrioid adenocarcinoma, and be used as a diagnostic tool for LVSI status.

Acknowledgements. We thank Sebastien Jacques and Florent Dumont (Plateforme Genomic, INSERM U1016, Institut Cochin), Annie Cortez (AC) (Department of Pathology, Tenon University Hospital) and Michèle Oster (UMRS 938) for expert contributions.

Disclosure/conflict of interest. Geoffroy Canlorbe received support from the Association pour la Recherche sur le Cancer (ARC). The authors declare no conflict of interest.

References

Agostini M. and Knight RA. (2014). miR-34: from bench to bedside. Oncotarget 5, 872-881.

Alcázar J.L., Galán M.J., Jurado M. and López-García G. (2002). Intratumoral blood flow analysis in endometrial carcinoma: correlation with tumor characteristics and risk for recurrence. Gynecol. Oncol. 84, 258-262.

Bendifallah S., Canlorbe G., Raimond E., Hudry D., Coutant C., Graesslin O., Touboul C., Huguet F., Cortez A., Daraï E. and Ballester M. (2014). A clue towards improving the European Society of Medical Oncology risk group classification in apparent early stage endometrial cancer? Impact of lymphovascular space invasion. Br. J. Cancer 110, 2640-2646.

Briët J.M., Hollema H., Reesink N., Aalders J.G., Mourits M.J., ten Hoor K.A., Pras E., Boezen H.M., van der Zee A.G. and Nijman H.W. (2005). Lymphvascular space involvement: an independent prognostic factor in endometrial cancer. Gynecol. Oncol. 96, 799-804.

Campos-Viguri G.E., Jiménez-Wences H., Peralta-Zaragoza O., Torres-Altamirano G., Soto-Flores D.G., Hernández-Sotelo D., Alarcón-Romero Ldel C., Jiménez-López M.A., Illades-Aguiar B. and Fernández-Tilapa G. (2015). miR-23b as a potential tumor suppressor and its regulation by DNA methylation in cervical cancer. Infect. Agent. Cancer 10, 42.

Canlorbe G., Wang Z., Laas E., Bendifallah S., Castela M., Lefevre M.,

Chabbert-Buffet N., Daraï E., Aractingi S., Méhats C. and Ballester M. (2016). Identification of microRNA expression profile related to lymph node status in women with early-stage grade 1-2 endometrial cancer. Mod. Pathol. 29, 391-401.

Castilla M.Á., Moreno-Bueno G., Romero-Pérez L., Van De Vijver K., Biscuola M., López-García M.Á., Prat J., Matías-Guiu X., Cano A., Oliva E. and Palacios J. (2011). Micro-RNA signature of the epithelial-mesenchymal transition in endometrial carcinosarcoma. J. Pathol. 223, 72-80.

Colombo N., Creutzberg C., Amant F., Bosse T., González-Martín A., Ledermann J., Marth C., Nout R., Querleu D., Mirza M.R. and Sessa C; ESMO-ESGO-ESTRO Endometrial Consensus Conference Working Group. (2016). ESMO-ESGO-ESTRO Consensus Conference on Endometrial Cancer: Diagnosis, Treatment and Follow-up. Int. J. Gynecol. Cancer. Off. J. Int. Gynecol. Cancer. Soc. 26, 2-30.

Díaz-López A., Moreno-Bueno G. and Cano A. (2014). Role of microRNA in epithelial to mesenchymal transition and metastasis and clinical perspectives. Cancer Manag. Res. 6, 205-216.

Donadelli M., Dando I., Fiorini C. and Palmieri M. (2014). Regulation of miR-23b expression and its dual role on ROS production and tumour development. Cancer Lett. 28;349, 107-113.

Farazi T.A., Hoell J.I., Morozov P. and Tuschl T. (2013). MicroRNAs in human cancer. Adv. Exp. Med. Biol. 774, 1-20.

Frumovitz M., Singh D.K., Meyer L., Smith D.H., Wertheim I., Resnik E. and Bodurka D.C. (2004). Predictors of final histology in patients with endometrial cancer. Gynecol. Oncol. 95, 463-468.

Fujii S., Kido A., Baba T., Fujimoto K., Daido S., Matsumura N., Konishi I. and Togashi K. I. (2015). Subendometrial enhancement and peritumoral enhancement for assessing endometrial cancer on dynamic contrast enhanced MR imaging. Eur. J. Radiol. 84, 581-589.

Guntupalli S.R., Zighelboim I., Kizer N.T., Zhang Q., Powell M.A., Thaker P.H., Goodfellow P.J. and Mutch D.G. (2012). Lymphovascular space invasion is an independent risk factor for nodal disease and poor outcomes in endometrioid endometrial cancer. Gynecol. Oncol. 124, 31-35.

Guo H., Ingolia N.T., Weissman J.S. and Bartel D.P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 466, 835-840.

Irizarry R.A., Hobbs B., Collin F., Beazer-Barclay Y.D., Antonellis K.J., Scherf U. and Speed T.P. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostat. Oxf. Engl. 4, 249-264.

Jiang X.F., Tang Q.L., Shen X.M., Li H.G., Chen L.H., Wang X.Y., Luo X., Lin Z.Q. and Jiang G.Y. (2012). Tumor-associated macrophages, epidermal growth factor receptor correlated with the triple negative phenotype in endometrial endometrioid adenocarcinoma. Pathol. Res. Pract. 208, 730-735.

Kitajima K., Suenaga Y., Ueno Y., Maeda T., Ebina Y., Yamada H., Okunaga T., Kubo K., Sofue K., Kanda T., Tamaki Y. and Sugimura K.I. (2015). Preoperative risk stratification using metabolic parameters of (18) F-FDG PET/CT in patients with endometrial cancer. Eur. J. Nucl. Med. Mol. Imaging 42, 1268-1275.

Kito M., Motoyama S., Fujita K., Miura M., Nanjo H., Sato N., Makino K., Sugawara T., Kato A., Tamura D., Takahashi K., Kumazawa Y., Sato W., Miura H., Shirasawa H., Sato A., Kumagai J. and Terada Y. (2015). CRP 1846C>T genetic polymorphism is associated with lymph node metastasis and/or severe lymphatic invasion in endometrial cancer. Tohoku. J. Exp. Med. 237, 25-30.

Kontomanolis E.N. and Koukourakis M.I. (2015). MicroRNA: The potential regulator of endometrial carcinogenesis. MicroRNA Shāriqah. United. Arab. Emir. 4, 18-25.

Kowalewska M., Bakula-Zalewska E., Chechlinska M., Goryca K., Nasierowska-Guttmejer A., Danska-Bidzinska A. and Bidzinski M. (2013). microRNAs in uterine sarcomas and mixed epithelial-mesenchymal uterine tumors: a preliminary report. Tumour Biol. 34, 2153-2160.

Kübler K., Ayub T.H., Weber S.K., Zivanovic O., Abramian A., Keyver-Paik M.D., Mallmann M.R., Kaiser C., Serçe N.B., Kuhn W. and Rudlowski C. (2014). Prognostic significance of tumor-associated macrophages in endometrial adenocarcinoma. Gynecol. Oncol. 135, 176-183.

Kurman R.J. (2014). WHO classification of tumours of female reproductive organs. 4th ed. International Agency for Research on Cancer. Lyon. p 307.

Lee H.J., Ahn B.C., Hong C.M., Song B.I., Kim H.W., Kang S., Jeong S.Y., Lee S.W. and Lee J. (2011). Preoperative risk stratification using (18) F-FDG PET/CT in women with endometrial cancer. Nukl. Nucl. Med. 50, 204-213.

Li F., Chen H., Huang Y., Zhang Q., Xue J., Liu Z. and Zheng F. (2015). miR-34c plays a role of tumor suppressor in HEC-1-B cells by targeting E2F3 protein. Oncol. Rep. 33, 3069-3074.

Liu T., Gao H., Yang M., Zhao T., Liu Y. and Lou G. (2014). Correlation of TNFAIP8 overexpression with the proliferation, metastasis, and disease-free survival in endometrial cancer. Tumour Biol. 35, 5805-5814.

Liu H., Wang X., Liu S., Li H., Yuan X., Feng B., Bai H., Zhao B., Chu Y. and Li H. (2016). Effects and mechanism of miR-23b on glucose-mediated epithelial-to-mesenchymal transition in diabetic nephropathy. Int. J. Biochem. Cell. Biol. 70, 149-160.

Majid S., Dar A.A., Saini S., Deng G., Chang I., Greene K., Tanaka Y., Dahiya R. and Yamamura S. (2013). MicroRNA-23b functions as a tumor suppressor by regulating Zeb1 in bladder cancer. PLoS One 8, e67686.

Majid S., Dar A.A., Saini S., Arora S., Shahryari V., Zaman M.S., Chang I., Yamamura S., Tanaka Y., Deng G. and Dahiya R. (2012). miR-23b represses proto-oncogene Src kinase and functions as methylation-silenced tumor suppressor with diagnostic and prognostic significance in prostate cancer. Cancer. Res. 72, 6435-6446.

Montserrat N., Mozos A., Llobet D., Dolcet X., Pons C., de Herreros A.G., Matias-Guiu X. and Prat J. (2012). Epithelial to mesenchymal transition in early stage endometrioid endometrial carcinoma. Hum. Pathol. 43, 632-643.

Morice P., Leary A., Creutzberg C., Abu-Rustum N. and Darai E. (2015). Endometrial cancer. Lancet. 387, 1094-1108.

Nougaret S., Reinhold C., Alsharif S.S., Addley H., Arceneau J., Molinari N., Guiu B. and Sala E. (2015). Endometrial cancer: Combined MR volumetry and diffusion-weighted imaging for assessment of myometrial and lymphovascular invasion and tumor grade. Radiology 276, 797-808.

O'Brien D.J., Flannelly G., Mooney E.E. and Foley M. (2009). Lymphovascular space involvement in early stage well-differentiated endometrial cancer is associated with increased mortality. Int. J. Obstet. Gynaecol. 116, 991-994.

Osmanağaoğlu M.A., Kadioğlu S., Osmanağaoğlu S., Bozkaya H., Reis A. and Tekelioğlu Y. (2005). The relationship between mutant p53

gene, DNA contents and conventional clinicopathological prognostic variables in cases with endometrial carcinoma. Eur. J. Gynaecol. Oncol. 26, 64-70.

Peinado H., Olmeda D. and Cano A. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat. Rev. Cancer 7, 415-428.

Pellegrino L., Krell J., Roca-Alonso L., Stebbing J. and Castellano L. (2013). MicroRNA-23b regulates cellular architecture and impairs motogenic and invasive phenotypes during cancer progression. Bioarchitecture 3, 119-124.

Sianou A., Galyfos G., Moragianni D., Andromidas P., Kaparos G., Baka S. and Kouskouni E. (2015). The role of microRNAs in the pathogenesis of endometrial cancer: a systematic review. Arch. Gynecol. Obstet. 292, 271-282.

Song T., Lee J.W., Choi C.H., Kim T.J., Bae D.S., Sung C.O., Song S.Y. and Kim B.G. (2012). Ploidy and S-phase fraction are correlated with lymphovascular space invasion that is predictive of outcomes in endometrial cancer. Int. J. Clin. Oncol. 17, 590-597.

Tomica D., Ramić S., Danolić D., Knezević F., Kolak T., Balja M.P., Alvir I., Mamić I. and Puljiz M. (2014). A correlation between the expression of estrogen receptors and progesterone receptors in cancer cells and in the myometrium and prognostic factors in endometrial cancer. Coll. Antropol. 38, 129-134.

Torres A., Torres K., Pesci A., Ceccaroni M., Paszkowski T., Cassandrini P., Zamboni G. and Maciejewski R. (2013). Diagnostic and prognostic significance of miRNA signatures in tissues and plasma of endometrioid endometrial carcinoma patients. Int. J. Cancer 132, 1633-1645.

Visser N.C., Bulten J., van der Wurff A.A., Boss E.A., Bronkhorst C.M., Feijen H.W, Haartsen J.E., van Herk H.A., de Kievit I.M., Klinkhamer P.J., Pijlman B.M., Snijders M.P., Vandenput I., Vos M.C., de Wit P.E., van de Poll-Franse L.V., Massuger L.F. and Pijnenborg J.M. (2015). PIpelle Prospective ENDOmetrial carcinoma (PIPENDO) study, pre-operative recognition of high risk endometrial carcinoma: a multicentre prospective cohort study. BMC Cancer 15, 487.

Wang L., Chen Y.J., Xu K., Xu H., Shen X.Z. and Tu R.Q. (2014). Circulating microRNAs as a fingerprint for endometrial endometrioid adenocarcinoma. PLoS One 9, e110767.

Wang P., Zhang J., Zhang L., Zhu Z., Fan J., Chen L., Zhuang L., Luo J., Chen H., Liu L., Chen Z. and Meng Z. (2013). MicroRNA 23b regulates autophagy associated with radioresistance of pancreatic cancer cells. Gastroenterology 145, 1133-1143.

Yamagami W., Susumu N., Tanaka H., Hirasawa A., Banno K., Suzuki N., Tsuda H., Tsukazaki K. and Aoki D. (2011). Immunofluorescence-detected infiltration of CD4+FOXP3+ regulatory T cells is relevant to the prognosis of patients with endometrial cancer. Int. J. Gynecol. Cancer. 21, 1628-1634.

Zhao L., Wang P., Liu Y., Ma J. and Xue Y. (2015a). miR-34c regulates the permeability of blood-tumor barrier via MAZ-mediated expression changes of ZO-1, occludin, and claudin-5. J. Cell. Physiol. 230, 716-731.

Zhao J., Liu T., Yu G. and Wang J. (2015b). Overexpression of HABP1 correlated with clinicopathological characteristics and unfavorable prognosis in endometrial cancer. Tumour Biol. 36, 1299-1306.

Accepted December 21, 2016