ISSN 2616-5791 Vol.3, Issue 1: 22-31, DOI: 10.25236/AJMHS.2022.030105

Mir-1251-5p/Mir-6892-5p Expression with Clinicopathological Factors in Premenopausal Endometrial Cancer

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Abstract: *Background: This study is aim to profile the differentially expressed microRNAs (DEMs) of premenopausal endometrial cancer (EC), identify their target genes and understand their roles in carcinogenesis. Methods: Next-generation sequencing (NGS) was performed on 3 premenopausal EC and 3 premenopausal normal endometrial tissues. Selection of candidate miRNAs and subsequent validation were performed by qRT-PCR on 20 premenopausal EC, 30 premenopausal normal endometrial and 40 postmenopausal EC samples. The relationship between DEMs and clinical characteristics was analyzed. Results: 136 upregulated and 131 downregulated DEMs were identified. The expression of miR-1251-5p was highly upregulated in premenopausal EC samples compared with premenopausal normal endometrial samples and significantly downregulated compared with postmenopausal EC samples. The expression of miR-6892-5p was highly upregulated in premenopausal EC samples compared with premenopausal normal endometrial samples and postmenopausal EC samples. In the premenopausal EC group, miR-1251-5p expression was closely correlated with menarche age, number of pregnancies, tumor grading, myometrial infiltration and lymph node metastasis; miR-6892-5p expression was closely correlated with BMI, hypertension, tumor grading, and metastasis. Conclusions: miR-1251-5p and miR-6892-5p may play important roles in tumorigenesis progression of premenopausal EC.*

Keywords: *Premenopausal endometrial cancer; differentially expressed microRNAs; next-generation sequencing; miR-1251-5p; miR-6892-5p*

1. Introduction

Endometrial cancer (EC) is one of the most common cancers of the female reproductive system. The incidence has risen by an approximate 50% since the early 1990s, and this trend is set to continue with a predicted incidence of 33/100,000 by 2035[1]. EC occurs predominantly among postmenopausal women, with a mean diagnosed age of 61 years; however, premenopausal EC is not rare. Approximately 20% of EC patients are premenopausal women, 5−30% are diagnosed before the age of 50 years old and 5% are diagnosed before the age of 40 years old [2]. Interestingly, some recent studies have reported that Asian women, including Asian native women and Asian immigrant women, are diagnosed with EC at a younger age than non-Asian women, and the proportion of Chinese women diagnosed with EC before menopause (38%) is higher than that of Western countries (<25%) [3]. Although the incidence of EC in young women has dramatically increased due to early-onset obesity and hyperinsulinemia in the last decade, there have been few studies on the clinical characteristics and pathogenesis related to premenopausal EC.

MicroRNAs (miRNAs) are small, single-stranded, noncoding RNAs composed of 19~25 nucleotides that play major roles in cell proliferation, cell death, metabolism, stem cell maintenance and differentiation and disease development in post-transcriptional gene regulation.

In this study, we aimed to identify differentially expressed miRNAs (DEMs) in premenopausal EC by next-generation sequencing (NGS) and qRT-PCR techniques. By identifying miRNA-gene expression signatures in premenopausal EC, we may provide new perspectives for the development of novel diagnostic methods, prognostic prediction tools, and therapeutic strategies for EC in younger women.

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2. Materials and methods

2.1. Patient Samples

The study was approved by the Ethics Committee of Shaanxi Provincial People's Hospital and was performed according to the principles of the Declaration of Helsinki. All experiments were performed in accordance with relevant guidelines and regulations, and informed consent was obtained from the participants. Tumor tissue samples were obtained from complete staging of endometrial carcinoma (bilateral salpingo-oophorectomy and pelvic and para-aortic lymphadenectomy) with previously untreated EC and were divided into premenopausal (pre-ca group) and postmenopausal (post-ca group) groups according to whether the patients were menopausal or not. All samples were histologically classified and graded by a clinical pathologist according to WHO guidelines. Healthy endometrial tissues (N group) were collected as negative controls from patients undergoing hysterectomy or curettage procedures for benign problems, such as endometrial hyperplasia and uterine myoma, and did not have a diagnosis of any type of cancer or prior cancer history.

Patient set 1 used for next-generation sequencing consisted of samples from 6 individuals (Table 1): 3 premenopausal EC tissue samples and 3 premenopausal normal endometrial tissue samples. For validation of the sequencing results, RNA samples from a second series of tumor and healthy tissues were prepared to examine miRNA expression via quantitative real-time PCR (qRT-PCR). Patient set 2 consisted of 90 samples (20 cases in the pre-ca group, 40 cases in the post-ca group and 30 cases in the N group) (Table 2).

Table 1: Clinical Characteristics of Patient Set 1.

Group	Number	age	stage	Grading(quantity)
pre-CA	Pre1	46	IA	G2
	Pre2	46	IB	G1
	Pre3	43	IIIA	G2
Normal	Normal1	47	-	-
	Normal2	47	-	-
	Normal3	49	-	-

Table 2: Clinical Characteristics of Patient Set 2.

		pre-CA	Normal	Post-Ca
Samples		20	30	40
Age(years)		43.70±6.86	45.80±6.17	58.25±5.94
Age at menarche (years)		13.10±1.62	13.14±1.10	14.97±1.25
Menstruation	Regularity	8	30	40
	Irregular	12	0	0
Pregnancies		2.35±1.31	2.64±1.28	2.23±1.25
Family history of cancer	Yes	2	1	3
	No	18	29	37
Hypertension	Yes	3	0	18
	No	17	30	22
Diabetes	Yes	2	0	9
	No	18	30	31
FIGO Stage	I	11	-	32
	II	5	-	4
	III	4	-	4
Grading (quantity)	G1	9	-	6
	G2	9	-	19
	G3	2	-	15
Myometrial invasion	<1/2	13	-	25
	≥1/2	7	-	15
Lymph node metastasis	Yes	2	-	4
	No	18	-	36

All samples were collected at the Department of Gynecology of Shaanxi Provincial People's Hospital, First Affiliated Hospital of Xi 'a Jiaotong University and Department of Female Tumor of Shaanxi Provincial Cancer Hospital from January 2017 to September 2019. Tissues were immediately snap-frozen in liquid nitrogen and then transferred to a -80 ℃ freezer following surgery.

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2.2. RNA Extraction, Small RNA Library Construction and Next-Generation Sequencing

Total RNA was extracted using a NAfast1000 Total RNA extraction kit (Piomeer, Xian, China) according to the manufacturer's instructions. RNA concentration and purity were quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) at O.D. 260 nm. RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent and Bioanalyzer 2100 System (Agilent Technologies, CA, USA) and RNA integrity number (RIN)>8.0 is the cutoff for RNA sequencing.

Sequencing libraries were generated according to the manufacturer's instructions using the NEBNext UltraTM small RNA Sample Library Prep Kit for Illumina (NEB, USA), and the index codes were added to the property sequence of each sample. Truseq PE Cluster KITV3-CBOT-HS (Illumina) was used to cluster the index-coded samples on the CBOT Cluster Generation System according to the manufacturer's instructions. After the cluster was generated, the library was prepared for sequencing, and reads were generated on the Illumina platform. Sequencing was performed at Biomarker Technologies (Beijing).

2.3. Bioinformatics and Data Processing

DEMs between premenopausal EC specimens and premenopausal normal endometrial tissue specimens were identified via the edgeR package. The resulting false discovery rate (FDR) was adjusted using the posterior probability of being DE (PPDE). An adjusted P-value<0.05 and a |FC|≥1.5 were the thresholds for significant differential expression.

2.4. Detection of DEMs

The quantification of miRNAs was performed by a two-step reaction using an Evo M-MLV RT Kit for qPCR (AG, China, Hunan) and a SYBR Green Premix Pro Taq HS qPCR kit (Pioneer, China, Xi'an) for validating the expression of miRNAs (Table 3). qPCR was carried out using an ABI7500 fluorescence quantitative PCR instrument (ABI, USA). Cycling conditions were as follows: 95℃ for 5 min, followed by 40 cycles at [95 ℃ for 20 sec, 50 ℃ for 30 sec, 72 ℃ for 30 sec, and 95 ℃ for 15 sec], 50 ℃ for 1 min, and 95 ℃ for 30 s. All reactions were performed in triplicate. All samples were analyzed using the endogenous reference RNA RNU6B. The fold changes in miRNA expression were calculated using the 2-ΔΔCt method, where ΔΔCt= (ΔCt target-ΔCt control) Sample2-(ΔCt target-ΔCt control) Sample1.

Table 3: Primer of qRT-PCR (5'-3').

miRNA	RT primer	F primer	R primer
hsa-miR-1269b	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCAGTA	ACACTCCAGCTGGGCTGGACTGAGCCATGC	TGGTGTCGTGGAGTCG
hsa-miR-1251-5p	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGCGCC	ACACTCCAGCTGGGACTCTAGCTGCCAAAG	TGGTGTCGTGGAGTCG
hsa-mir-6744-3p	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTGCAGGA	ACACTCCAGCTGGGGGGCCTCTCTTGTCATC	TGGTGTCGTGGAGTCG
hsa-miR-133a-5p	CTCAACTGGGTGTCGTGGAGTCGGGCAATTCAGTTGAGATTTGG	ACACTCCAGCTGGGAGCTTGGTAAAATGGAA	TGGTGTCGTGGAGTCG
hsa-miR-1258	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTTCCAC	ACACTCCAGCTGGGAGTTAGGATTAGGTCG	TGGTGTCGTGGAGTCG
hsa-miR-4455	CTCAACTGGTGTTCGTGGAGTCGGCAATTCAGTTGAGAAAAAACAC	ACACTCCAGCTGGGAGGGTGTGTGTGTTT	TGGTGTCGTGGAGTCG
hsa-miR-371a-5p	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGTGCCC	ACACTCCAGCTGGGACTCAAACTGTGGGGG	TGGTGTCGTGGAGTCG
hsa-miR-4521	CTCAACTGGTGTCGTGGAGTCGGGCAATTCAGTTGAGCTGAGC	ACACTCCAGCTGGGGCTAAGGAAGTCCTGT	TGGTGTCGTGGAGTCG
hsa-miR-6765-3p	CTCAACTGGTGTTCGTGGAGTCGGCAATTCAGTTGAGCTGGGC	ACACTCCAGCTGGGTCACCTGGCTGGCCC	TGGTGTCGTGGAGTCG
hsa-miR-6892-5p	CTCAACTGGTTCGTGGAGTCGGCAATTCAGTTGATTCCTAC	ACACTCCAGCTGGGGTAAGGGACCGGAGA	TGGTGTCGTGGAGTCG
Endogenous reference RNA			
U6	-	CTCGCTTCGGCAGCACATATACT	ACGCTTCACGAATTTGCGTGTC

2.5. Statistical Analyses

The miRNA expression data were statistically analyzed using the Statistical Product and Service Solutions (SPSS) Statistical Package, Version 23.0 (Chicago, Illinois). All measurement data are expressed as the mean+s.d., and $\chi 2$ test was used for counting data. Student's t-test or the Mann-Whitney

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U test was used to compare two groups, according to the result of the Shapiro-Wilk normality test. All statistical tests were two-sided, and P-values<0.05 were considered significant.

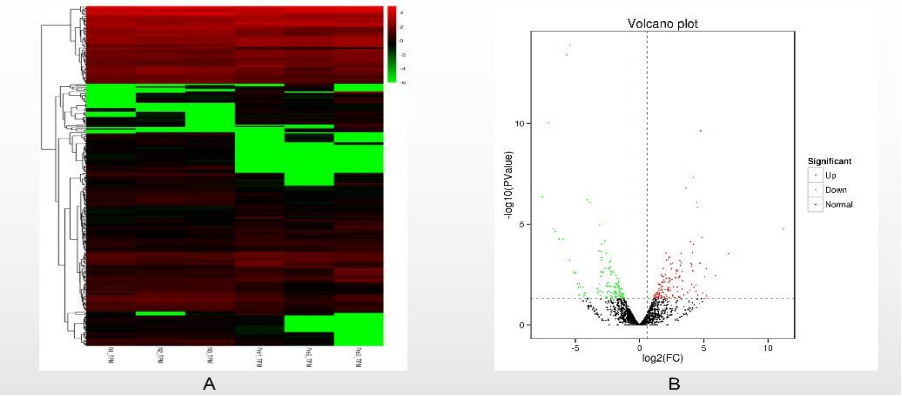
3. Results

3.1. Analysis of DEMs

A total of 2551 miRNAs were obtained, of which 1711 were known miRNAs and 840 were newly predicted miRNAs. In total, 267 miRNAs with a threshold of P<0.05 and |log2FC| were considered significantly DE, 136 of which were upregulated and 131 of which were downregulated. The top 20 upregulated genes and top 20 downregulated genes are shown in Table 4. The differences in miRNA expression levels between the two groups of samples were observed via the MA map cluster diagram and volcano plot (Figure 1).

Table 4: List of top 20 up-regulated and top 20 down-regulated DEGs between premenopausal EC tissue samples and premenopausal normal endometrial tissue samples.

Up-regulated			Down-regulated		
miRNA-ID_LIST	PValue	log2FC	miRNA-ID_LIST	PValue	log2FC
hsa-miR-1269b	1.72×10^{-5}	11.19	novel-miR-432	4.33×10^{-7}	-7.56
novel-miR-221	2.95×10^{-4}	6.94	novel-miR-713	4.58×10^{-7}	-7.56
hsa-miR-1251-5p	0.04	5.94	hsa-miR-4455	9.59×10^{-11}	-7.11
hsa-miR-6744-3p	0.04	5.22	novel-miR-569	1.73×10^{-5}	-6.72
hsa-miR-133a-5p	0.02	5.21	novel-miR-630	2.34×10^{-5}	-6.55
hsa-miR-1258	4.86×10^{-10}	5.09	novel-miR-189	5.48×10^{-5}	-6.28
hsa-miR-1323	4.05×10^{-3}	5.08	hsa-miR-371a-5p	5.56×10^{-5}	-5.96
novel-miR-619	2.62×10^{-2}	4.93	novel-miR-192	4.01×10^{-14}	-5.67
hsa-miR-499a-5p	4.50×10^{-5}	4.85	novel-miR-226	6.38×10^{-4}	-5.45
hsa-miR-1-3p	2.34×10^{-10}	4.76	hsa-miR-4521	1.27×10^{-14}	-5.43
hsa-miR-5571-3p	8.69×10^{-4}	4.71	novel-miR-853	2.39×10^{-3}	-5.13
hsa-miR-133b	0.03	4.54	novel-miR-235	2.67×10^{-3}	-4.99
hsa-miR-146a-3p	1.52×10^{-6}	4.53	novel-miR-102	9.22×10^{-3}	-4.83
hsa-miR-205-5p	8.20×10^{-7}	4.45	novel-miR-101	9.23×10^{-3}	-4.83
hsa-miR-6744-5p	0.03	4.41	novel-miR-46	0.01	-4.74
novel-miR-454	0.02	4.39	novel-miR-220	0.03	-4.72
novel-miR-434	0.02	4.39	hsa-miR-6765-3p	8.95×10^{-3}	-4.68
novel-miR-180	0.01	4.24	novel-miR-43	0.01	-4.63
novel-miR-688	9.99×10-5	4.20	novel-miR-113	0.01	-4.40
hsa-miR-133a-3p	4.78×10-8	4.20	hsa-miR-6892-5p	0.04	-4.36



Note: (A) Different rows represent different miRNAs, color represents the level of expression of miRNAs in sample quantity log10 (miRNA + 0.000001). (B) Each point in the figure represents a miRNA. The abscissa represents the logarithm of the differential multiple of the expression of a miRNA in two samples and the y-coordinate is the negative logarithm of pvalue. The green points in the figure represent down-regulated differentially expressed miRNAs, the red points represent up-regulated differentially expressed miRNAs, and the black points represent non-differentially expressed miRNAs.

Figure 1: Heatmap(A) and Volcano Plot(B) of differentially expressed miRNAs between 3 premenopausal EC tissue samples and 3 premenopausal normal endometrial tissue samples

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3.2. Known DEMs in EC Identified by Sequencing and qRT-PCR

The qRT-PCR results showed that although the melt curves of miR-6744-3p, hsa-miR-1258 and hsa-miR-371a-5p displayed no specificity, those of the other 7 miRNAs were all specific. Through additional analysis, we found that the above 7 miRNAs had significant differences in expression between normal endometrial tissues and EC (including premenopausal EC and postmenopausal EC) tissues; except for miR-133a-5p, the expression of the other 6 miRNAs between premenopausal EC and normal endometrial tissues was also significantly different, while there were three significant DEMs (miR-1251-5p, miR-133a-5p and miR-6892-5p) between the premenopausal EC and postmenopausal EC groups, and the expression of two genes (miR-1251-5p and miR-6892-5p) was significantly different between premenopausal EC tissues and normal endometrium and between premenopausal EC and postmenopausal EC tissues (Table 5, Figure 2).

Table 5: The relative expression levels of candidate miRNAs in different groups of endometrial tissues (Values for each gene were normalized to the level of endogenous gene, U6snRNA).

Candidate miRNAs	Pre-ca	Normal	Post-ca	P (All-ca Vs. Normal)	P (Pre-ca Vs. Normal)	P (Pre-ca Vs. Post-ca)
miR-1269b	$1.57\times10^{-2}\pm3.35\times10^{-2}$	$1.94\times10^{-3}\pm3.28\times10^{-3}$	$3.22\times10^{-2}\pm5.17\times10^{-2}$	0.000	0.000	0.766
miR-1251-5p	$8.51\times10^{-3}\pm1.43\times10^{-2}$	$4.81\times10^{-4}\pm4.22\times10^{-4}$	$1.26\times10^{-2}\pm1.58\times10^{-2}$	0.000	0.028	0.048
miR-133a-5p	$1.17\times10^{-3}\pm3.56\times10^{-3}$	$1.52\times10^{-4}\pm2.01\times10^{-4}$	$1.37\times10^{-3}\pm2.24\times10^{-3}$	0.000	0.122	0.009
miR-4455	0.82 ± 0.52	0.15 ± 0.12	1.96 ± 2.22	0.000	0.000	0.151
miR-4521	$5.61\times10^{-3}\pm5.47\times10^{-3}$	$1.51\times10^{-3}\pm2.97\times10^{-3}$	$7.84\times10^{-3}\pm1.26\times10^{-2}$	0.000	0.001	0.904
miR-6765-3p	0.33 ± 0.54	$7.27\times10^{-2}\pm9.85\times10^{-2}$	0.77 ± 1.46	0.000	0.000	0.344
miR-6892-5p	$3.69\times10^{-3}\pm8.06\times10^{-3}$	$1.11\times10^{-4}\pm1.41\times10^{-4}$	$3.27\times10^{-3}\pm5.22\times10^{-3}$	0.000	0.000	0.033

(Mann-Whitney U test)

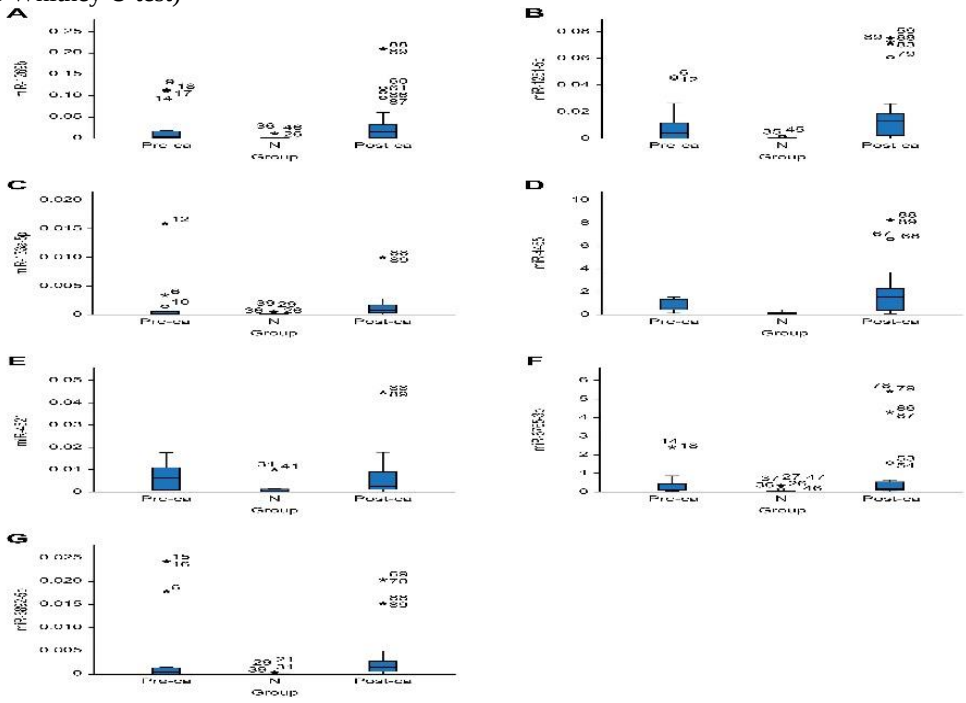

Figure 2: The screened miRNAs were relatively expressed in different groups of endometrial tissues.

3.3. Target Gene Prediction

To explore the biological function of the DEMs identified in this analysis, computational analysis was performed using two independent experimentally confirmed databases, miRanda and TargetScan, to analyze the target RNAs of miR-1251-5p and miR-6892-5p (Table 6).

Table 6: The target RNAs related to miR-1251-5p and miR-6892-5p.

#ID	Target
miR-1251-5p	RFPL1; GALNT17; ZNF736; CCDC153; RFPL2; PIGV; AC139530.2; RILPL2; PPT2-EGFL8; TMEM132A; FANK1; SPRYD4; PMEL; PURG; DR1; EYS; PLEKHM3; MRPL12; FBXO30; LZTS2; DNAH17

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miR-6892-5p	LHX9; NDUFB3; FRK; C1QTNF7; INSRR; SOD2; DNHD1; GIGYF1; CADM3; KAT14; HCK; RABL3; PET117; CCDC80; IL17C; C12orf45; SCARB1; PKLR; HEATR1; SYT16; HAPLN3; TOR1AIP2; ERO1A; C2CD6; GNB5; NDUFB5; CHCHD5

3.4. DEMs and Clinical Factors

In the premenopausal EC group, the expression of miR-1251-5P was significantly different in menarche age (P=0.017), number of pregnancies (P=0.000), tumor cell differentiation level (P=0.031), myometrial infiltration (P=0.046), and lymph node metastasis (P=0.011), and the expression of miR-6892-5P was significantly different in BMI (P=0.004), hypertension (P=0.006), tumor cell differentiation level (P=0.006), and metastasis (including cervical uteri, lymph node and other organ metastases) (P=0.005). In the postmenopausal EC group, there was no significant difference in miR-1251-5p expression in any of the factors above except diabetes (P=0.031), and there was no significant difference in miR-6892-5p expression in any of the factors above except FIGO stage (P=0.040) (Tables 7, 8).

Table 7: Relationship between miR-1251-5p expression and clinic pathological features of EC patients.

Variables	miR-1251-5p					
	Premenopausal EC			Postmenopausal EC		
	Samples	Relative expression	P	Samples	Relative expression	P
Age at menarche (years)						
≤12	8	$6.76\times10^{-3}\pm1.56\times10^{-2}$	0.017	9	$1.63\times10^{-2}\pm2.20\times10^{-2}$	0.526
>12 ~ ≤14	8	$1.75\times10^{-2}\pm1.46\times10^{-2}$		9	$9.42\times10^{-3}\pm6.80\times10^{-3}$	
>14	4	$1.37\times10^{-3}\pm1.54\times10^{-3}$		22	$2.14\times10^{-2}\pm2.48\times10^{-2}$	
Menstrual cycle						
Regularity	8	$9.25\times10^{-3}\pm1.52\times10^{-2}$	0.678	40	*-	
Irregularity	12	$7.25\times10^{-3}\pm1.41\times10^{-2}$		0		
BMI						
<24	3	$6.62\times10^{-3}\pm6.79\times10^{-3}$	0.094	10	$2.49\times10^{-2}\pm2.62\times10^{-2}$	0.356
≥24~<28	4	$1.76\times10^{-2}\pm1.01\times10^{-2}$		27	$1.42\times10^{-2}\pm1.85\times10^{-2}$	
≥28	13	$8.37\times10^{-3}\pm1.67\times10^{-2}$		3	$2.33\times10^{-2}\pm3.27\times10^{-3}$	
Pregnancies						
0 ~1	5	$4.42\times10^{-6}\pm6.05\times10^{-6}$	0.000	12	$1.27\times10^{-2}\pm1.96\times10^{-2}$	0.092
≥2	15	$1.33\times10^{-2}\pm1.55\times10^{-3}$		28	$1.97\times10^{-2}\pm2.24\times10^{-2}$	
Family history of cancer						
Yes	2	$3.71\times10^{-5}\pm1.41\times10^{-6}$	0.312	3	$1.28\times10^{-2}\pm5.18\times10^{-3}$	0.734
No	18	$1.11\times10^{-3}\pm1.50\times10^{-3}$		37	$1.80\times10^{-2}\pm2.24\times10^{-2}$	
Hypertension						
Yes	3	$2.10\times10^{-2}\pm2.10\times10^{-2}$	0.093	18	$1.85\times10^{-2}\pm2.47\times10^{-2}$	0.644
No	17	$8.01\times10^{-3}\pm1.31\times10^{-2}$		22	$1.68\times10^{-2}\pm1.92\times10^{-2}$	
Diabetes						
Yes	2	$1.10\times10^{-5}\pm7.10\times10^{-9}$	0.168	9	$3.41\times10^{-2}\pm2.94\times10^{-2}$	0.031
No	18	$1.11\times10^{-2}\pm1.50\times10^{-2}$		31	$1.28\times10^{-2}\pm1.64\times10^{-2}$	
FIGO stage						
I	11	$8.22\times10^{-3}\pm1.01\times10^{-2}$	0.206	32	$1.82\times10^{-2}\pm2.15\times10^{-2}$	0.501
II	5	$1.27\times10^{-2}\pm1.87\times10^{-2}$		4	$5.43\times10^{-3}\pm3.00\times10^{-3}$	
III	4	$1.13\times10^{-2}\pm2.26\times10^{-2}$		4	$2.49\times10^{-2}\pm3.15\times10^{-2}$	
Grading						
G1	9	$1.10\times10^{-2}\pm9.45\times10^{-3}$	0.031	6	$1.11\times10^{-2}\pm8.93\times10^{-3}$	0.638
G2	9	$1.10\times10^{-3}\pm2.00\times10^{-2}$		19	$2.25\times10^{-2}\pm2.62\times10^{-2}$	
G3	2	$2.90\times10^{-9}\pm1.00\times10^{-10}$		15	$1.40\times10^{-2}\pm1.79\times10^{-2}$	
Myometrial infiltration						
<1/2	13	$1.12\times10^{-2}\pm1.38\times10^{-2}$	0.046	25	$1.99\times10^{-2}\pm2.34\times10^{-2}$	0.476
≥1/2	7	$7.72\times10^{-3}\pm1.68\times10^{-2}$		15	$1.38\times10^{-2}\pm1.82\times10^{-2}$	
Lymph node metastasis						
Yes	2	$4.56\times10^{-2}\pm7.07\times10^{-4}$	0.011	4	$2.49\times10^{-2}\pm3.15\times10^{-2}$	0.983
No	18	$6.00\times10^{-3}\pm8.40\times10^{-4}$		36	$1.68\times10^{-2}\pm1.07\times10^{-2}$	
Tumor metastasis (including cervix uteri, lymph node and another organs metastasis)						
Yes	9	$1.21\times10^{-2}\pm1.91\times10^{-2}$	0.824	9	$1.38\times10^{-2}\pm2.21\times10^{-2}$	0.292
No	11	$8.22\times10^{-3}\pm1.01\times10^{-2}$		31	$1.87\times10^{-2}\pm2.16\times10^{-2}$	

(Note: * The menstrual cycle of all the postmenopausal women is regular. Mann-Whitney U test, $\bar{x}\pm s$)

Table 8: Relationship between miR-6892-5p expression and clinic pathological features of EC patients.

Variables	miR-6892-5p	
	Premenopausal EC	Postmenopausal EC

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	Samples	Relative expression	P	samples	Relative expression	P
Age at menarche (years)						
≤12	8	$2.51\times10^{-3}\pm6.22\times10^{-3}$	0.511	9	$1.39\times10^{-3}\pm1.60\times10^{-3}$	0.294
>12 ~ ≤14	8	$6.64\times10^{-3}\pm1.09\times10^{-2}$		9	$5.24\times10^{-3}\pm8.57\times10^{-3}$	
>14	4	$5.59\times10^{-4}\pm5.41\times10^{-4}$		22	$3.14\times10^{-3}\pm4.16\times10^{-3}$	
Menstrual cycle						
Regularity	8	$2.78\times10^{-3}\pm6.13\times10^{-3}$	0.347	40	*-	
Irregularity	12	$4.44\times10^{-3}\pm9.29\times10^{-3}$		0		
BMI						
<24	3	$1.20\times10^{-3}\pm2.97\times10^{-4}$	0.004	10	$1.37\times10^{-3}\pm9.40\times10^{-4}$	0.638
≥24~<28	4	$1.28\times10^{-2}\pm1.32\times10^{-2}$		27	$3.97\times10^{-3}\pm6.11\times10^{-3}$	
≥28	13	$1.58\times10^{-3}\pm4.90\times10^{-3}$		3	$2.56\times10^{-3}\pm2.10\times10^{-3}$	
Pregnancies						
0 ~1	5	$3.26\times10^{-4}\pm3.30\times10^{-4}$	0.092	12	$1.56\times10^{-3}\pm1.93\times10^{-3}$	0.061
≥2	15	$4.02\times10^{-3}\pm9.04\times10^{-3}$		28	$3.93\times10^{-3}\pm5.93\times10^{-3}$	
Family history of cancer						
Yes	2	$8.97\times10^{-5}\pm1.40\times10^{-9}$	0.443	3	$1.84\times10^{-3}\pm2.72\times10^{-3}$	0.626
No	18	$4.18\times10^{-3}\pm8.38\times10^{-3}$		37	$3.33\times10^{-3}\pm5.32\times10^{-3}$	
Hypertension						
Yes	3	$2.22\times10^{-2}\pm3.70\times10^{-3}$	0.006	18	$5.19\times10^{-3}\pm7.17\times10^{-3}$	0.369
No	17	$5.30\times10^{-4}\pm5.40\times10^{-4}$		22	$1.60\times10^{-3}\pm1.30\times10^{-3}$	
Diabetes						
Yes	2	$6.87\times10^{-4}\pm1.50\times10^{-9}$	0.798	9	$5.45\times10^{-3}\pm5.81\times10^{-3}$	0.083
No	18	$4.12\times10^{-3}\pm8.41\times10^{-2}$		31	$2.57\times10^{-3}\pm4.87\times10^{-3}$	
FIGO stage						
I	11	$5.14\times10^{-3}\pm9.48\times10^{-3}$	0.206	32	$2.30\times10^{-3}\pm3.60\times10^{-3}$	0.040
II	5	$1.72\times10^{-4}\pm1.18\times10^{-4}$		4	$1.15\times10^{-2}\pm1.01\times10^{-2}$	
III	4	$4.54\times10^{-3}\pm8.90\times10^{-3}$		4	$2.25\times10^{-3}\pm2.52\times10^{-3}$	
Grading						
G1	9	$6.17\times10^{-3}\pm1.03\times10^{-2}$	0.008	6	$6.21\times10^{-4}\pm6.24\times10^{-4}$	0.052
G2	9	$2.20\times10^{-3}\pm5.89\times10^{-3}$		19	$2.92\times10^{-3}\pm4.49\times10^{-3}$	
G3	2	$8.57\times10^{-5}\pm1.00\times10^{-10}$		15	$4.63\times10^{-3}\pm6.55\times10^{-3}$	
Myometrial infiltration						
<1/2	13	$2.44\times10^{-3}\pm6.59\times10^{-3}$	0.841	25	$3.84\times10^{-3}\pm6.34\times10^{-3}$	0.834
≥1/2	7	$6.26\times10^{-3}\pm1.03\times10^{-2}$		15	$2.17\times10^{-3}\pm1.82\times10^{-3}$	
Lymph node metastasis						
Yes	2	$8.99\times10^{-3}\pm1.26\times10^{-2}$	0.949	4	$2.25\times10^{-3}\pm2.52\times10^{-3}$	0.652
No	18	$3.20\times10^{-3}\pm7.69\times10^{-3}$		36	$3.32\times10^{-3}\pm5.39\times10^{-3}$	
Tumor metastasis (including cervix uteri, lymph node and another organs metastasis)						
Yes	9	$2.11\times10^{-3}\pm5.92\times10^{-3}$	0.005	9	$6.27\times10^{-3}\pm8.14\times10^{-3}$	0.178
No	11	$5.14\times10^{-3}\pm9.48\times10^{-3}$		31	$2.33\times10^{-3}\pm3.66\times10^{-3}$	

(Note: * The menstrual cycle of all the postmenopausal women is regular. Mann-Whitney U test, $x \pm s$.

Comparisons of the clinical and pathological characteristics between premenopausal and postmenopausal EC patients showed that the age of menarche of premenopausal EC patients was significantly earlier than that of postmenopausal EC patients, and the proportion of patients with irregular menstrual cycle and obesity was significantly higher than that of postmenopausal EC patients. However, the proportion of postmenopausal EC patients with hypertension and poor tissue differentiation was significantly higher than that of premenopausal EC patients (Table 9).

Sixty EC patients were followed up to 2020-10, with the longest follow-up time of 34 months and the shortest follow-up time of 12 months. There were 3 cases of recurrent disease, all of which were postmenopausal EC. One patient (stage IB, G3) who did not undergo postoperative radiotherapy due to postoperative urine retention refused treatment after lung metastasis occurred 13 months after surgery (death). One patient (stage IB, G1) had rectal metastasis recurrence 17 months after surgery and one patient (stage IIIC1, G1) developed distant metastasis and recurrence to the peritoneum and omentum 14

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months after surgery. Both of these patients are currently under treatment. There was no correlation between recurrence and miR-1251-5p or miR-6892-5p expression levels (P=0.501, P=956).

Table 9: Clinical and pathological features between Premenopausal EC and Postmenopausal EC.

Variables		Premenopausal EC (20 cases)	Postmenopausal EC (40 cases)	P
Age at menarche[1](years)		13.10±1.62	14.97±1.25	0.000
menstrual cycle[2]	Regularity	8(40%)	40(100%)	0.000
	Irregularity	12(60%)	0(0%)	
Pregnancies[1]		2.35±1.31	2.23±1.25	0.062
BMI[2]	<24	3(15%)	10(25%)	0.000
	≥24~<28	4(20%)	27(67.5%)	
	≥28	13(65%)	3(7.5%)	
Family history of cancer[2]	Yes	2(10%)	3(7.5%)	1.000
	No	18(90%)	37(92.5%)	
Hypertension[2]	Yes	3(15%)	18(45%)	0.022
	No	17(85%)	22(55%)	
Diabetes[2]	Yes	2(10%)	9(22.5%)	0.409
	No	18(90%)	31(77.5%)	
FIGO Stage[1]	I	11(55%)	32(80%)	0.052
	II	5(25%)	4(10%)	
	III	4(20%)	4(10%)	
Grading[1]	G1	9(45%)	6(15%)	0.004
	G2	9(45%)	19(47.5%)	
	G3	2(10%)	15(37.5%)	
Myometrial infiltration[2]	<1/2	13(65%)	25(62.5%)	0.850
	≥1/2	7(35%)	15(37.5%)	
Lymph node metastasis[2]	Yes	2(10%)	4(10%)	1.000
	No	18(90%)	36(90%)	
Recurrence[2]		3(5%)	0	0.492

(Note: 1. Mann-Whitney U test, 2. Pearson Chi-square test.)

4. Discussion

It is well known that 80%-85% of ECs are endometrioid adenocarcinomas associated with estrogen dependence. Non-resistant estrogen exposure is an important risk factor; long-term exposure leads to persistent endometrial hyperplasia and even progression to cancer. Before menopause, estrogen and progesterone are primarily synthesized in the ovaries, although estrogens are also produced via the peripheral testosterone aromatization, especially in obese women. During the transition to the perimenopausal period, ovarian failure gradually leads to a shift in the balance of these two hormones toward more unopposed extraglandular production of estrogen in the body, which may lead to differences in risk factors and pathogenesis between premenopausal and postmenopausal women. In recent years, accumulating evidence has shown that menopause and older age might be a risk factor for malignancy of endometrial polyps and abnormal uterine bleeding (AUB)[4], whereas premenopausal EC is more closely associated with obesity[5], polycystic ovary syndrome (PCOS), nulliparity, and diabetes [6-7]. Our study confirmed the findings that among premenopausal EC patients, the proportion of obese and irregular menstrual cycle patients was significantly higher than that of postmenopausal EC patients. These studies suggest that in addition to hormonal factors, insulin also plays a significant role in the carcinogenesis of EC in premenopausal women. In addition, the differences in pathologic characteristics and prognosis for premenopausal EC and postmenopausal EC vary in different studies. Several previous studies have reported an incidence of concurrent ovarian cancer in young EC women ranging from 7% to 29% and recommended caution when considering ovarian preservation in young EC patients [8]. However, other studies have shown that young women with EC often have low-grade, early-stage tumors and have a more favorable prognosis than older patients and suggest that ovarian preservation in premenopausal women with early-stage EC may be safe and not associated with an increase in cancer-related mortality [2,9]. Our study found that premenopausal EC had good grading (P=0.004) and a lower recurrence rate (0 vs. 5%) than postmenopausal EC, although there was no statistically significant difference in the recurrence rate between the two groups due to the small number of cases and short follow-up time. This discrepancy may be due to differences in sample sizes, the characteristics of patients, and study regions. These studies identified differences in ECs between premenopausal and postmenopausal women, suggesting that there may be differences in tumor biology between the two

ISSN 2616-5791 Vol.3, Issue 1: 22-31, DOI: 10.25236/AJMHS.2022.030105

groups.

Abnormal miRNA expression leads to the occurrence of certain diseases and even carcinogenesis [10-11], including endometrial cancer [12]. Some studies have found that miRNA expression is associated with prognostic factors, including lymph node involvement, lymphovascular space invasion (LVSI), recurrence-free survival (RFS) and overall survival (OS) [13]. This study investigated differences in the miRNA gene profile for premenopausal EC by next-generation sequencing techniques and identified a total of 267 DEMs between premenopausal EC and normal endometrial tissues. To our knowledge, this is the first report on the miRNA expression profile of premenopausal EC. Improved knowledge of the miRNA expression profile may explain molecular mechanisms involved in premenopausal EC and may serve as a basis for the development of diagnostic and prognostic indicators or the identification of potential new therapeutic targets.

miR-1251-5p is located on chromosome 12q23.1. Previously, some studies showed that miR-1251-5p expression was downregulated in stomach carcinoma cells and clear cell renal cell carcinoma [14]. Other studies found that miR-1251-5p, a tumor driver, promotes malignant behavior and autophagy of ovarian cancer cells by targeting the tumor suppressor gene TBCC and promotes tumor growth and metastasis of liver cancer by targeting AKAP12[15-16]. These studies indicate that miR-1251-5p biological functions in different carcinomas are diverse. Our experiments also show that miRNA-1251-5p expression was highly upregulated compared with that in premenopausal normal endometrial samples and significantly downregulated compared with that in postmenopausal EC samples. Additionally, in the pre-ca group, its expression was significantly upregulated in cancer tissues of patients with early menarche, low fertility, poor differentiation and deep muscular invasion compared with patients with late menarche, well differentiated tumors and shallow muscular invasion, but no such associations (except diabetes) were observed in postmenopausal EC, suggesting that miR-1251-5p may be a key gene affecting the occurrence and invasion of premenopausal EC. However, miR-1251-5p expression was significantly decreased in premenopausal EC patients with lymph node positivity compared with negative patients, which may be related to the relatively small number of lymph node metastases. These relationships require further verification with a large sample.

There have been no reports on miR-6892-5p. This study showed that miR-6892-5p expression was highly upregulated in premenopausal EC samples compared premenopausal normal endometrial and postmenopausal EC samples. In the premenopausal EC group, miR-6892-5p expression was closely correlated with tumor cell differentiation level and metastasis. We used miRanda and TargetScan to analyze the target RNAs of miR-6892-5p. Some of these target genes, such as HEATR1 and FRK, have been reported to act as tumor suppressor genes in different tumors[17-19]. Some of these target genes, including CCDC80, NDUFB3 and NDUFB5, play an important protective role in glycolipid metabolism [20-21]. Our study found that miR-6892-5p expression was significantly different in the endometrial cancer tissues of premenopausal EC between obese, overweight and normal populations. Based on these findings, it can be concluded that miR-6892-5p may be associated with the tissue characteristics and carcinogenesis of premenopausal EC by regulating insulin metabolism and affecting fat distribution through these target genes. The target RNAs and their related functions need to be further studied in subsequent experiments.

5. Conclusions

In conclusion, miR-1251-5p and miR-6892-5p may play important roles in the occurrence and development of premenopausal EC. However, due to the relatively limited number of sequencing samples and verification samples in this experiment, these findings need to be further verified in larger samples. Their target RNAs and signaling pathways related to premenopausal EC also need to be further studied.

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