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Diagnostic and prognostic potential of microRNA profiles in endometrioid endometrial cancer

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Gene regulation is influenced by microRNAs (miRNAs), a class of non-coding RNAs currently being studied as biomarkers for various diseases. miRNAs, which act as regulators of gene expression and potential biomarkers, were profiled in endometrioid type endometrial cancer (EEC). 28 miRNAs were selected based on their role in regulating EEC-related oncogenes and tumor suppressors (e.g., PTEN, KRAS, β-CATENIN), maintaining tissue stability, and their previous associations with endometrial cancer. This study investigated the expression of miRNAs and their association with key signalling pathways (PI3K/AKT, RAS/MAPK, Wnt/β-Catenin) and their potential as diagnostic biomarkers for EEC. The study also investigated the relationship between miRNA regulation, endometrial pathology, and PTEN, KRAS, and β-CATENIN mRNA expression levels. Women who had received an EEC diagnosis participated in a 14-month prospective cohort study at Gazi University Hospital. Samples were obtained from patients with EEC during frozen sections and healthy women who underwent hysterectomy for benign disease. qPCR examined the miRNA and mRNA levels. 97 women participated, comprising 47 EEC patients and 50 healthy controls. The association was identified between miRNA expression levels and cancer grade. As the tumor grade increases, the expression of miRNAs (miR-let-7c, miR-18a-3p, miR-21, miR-30b, miR-96, miR-130a, miR-141, miR-181b, miR-182, miR-183, miR-2001, miR-200b, miR-200c, miR-203, miR-205, and miR-429) gradually increases. Conversely, twelve miRNAs demonstrated relative expression during the transition from normal endometrium (NE) to EEC (miR-let-7c, miR-let-7e, miR-30c, miR-101, miR-125b, miR-126, miR-129-2, miR-217, miR-324-3p, miR-518b, miR-543, and miR-596). miRNAs could serve as valuable biomarkers for both early detection of EEC and for distinguishing between different tumor grades. We showed that miRNAs have good diagnostic sensitivity for identifying EEC and EC grading. In addition, miRNA improved the ability to discriminate between ECs of different grades.

Keywords Endometrioid-type endometrial cancer, MicroRNAs, PTEN, KRAS, β-CATENIN

Abbreviations

EC	Endometrium Cancer
NE	Normal Endometrium
EEC	Endometrioid-type Endometrial Cancer
miRNA	MicroRNA
MMR	Mismatch Repair
MSI	Microsatellite Instability
RT-PCR	Real-time Polymerase Chain Reaction
FIGO	International Federation of Obstetrics and Gynecology
hMLH1	Human mutL Homolog 1
MSH	mutS Homolog

The prevalence of endometrial cancer (EC), the most common malignancy of the female reproductive system, is increasing due to factors such as an ageing population and rising obesity rates[1,2]. The most common histological

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subtype is endometrioid type endometrial cancer (EEC). The standard of care is total hysterectomy and bilateral salpingo-oophorectomy. However, the clinical benefit of lymph node dissection inpatients with early-stage disease remains controversial, and although it is one of the tumours with the best prognosis, some patients may be over- or under-treated, and although it is one of the tumours with the best prognosis, some patients may be over- or under-treated. Despite the molecular classification and biomarkers used in EC, the prediction of recurrence and metastasis has not become standard in clinical practice.

Gene silencing is achieved by microRNAs (miRNAs) by targeting complementary mRNA sequences. These non-coding RNAs, 19–23 nucleotides in length, are thought to control approximately 30% of the human genome[3]. These non-coding RNAs exert their regulatory function by binding to specific mRNA targets with complementary sequences. This post-transcriptional regulation occurs via translational repression or mRNA degradation. The role of microRNAs in carcinogenesis is being investigated because the molecules are expressed at different levels in different tissues. In addition, the levels of different microRNAs are highly variable in malignant tissues[4]. miRNAs can acquire oncogenic or tumor-suppressor properties depending on the characteristics of the mRNAs they target in molecular pathways.

EECs with microsatellite instability (MSI) can exhibit several genetic alterations, including mutations in the PTEN, KRAS, and β-catenin genes[5–8]. The mechanisms involved in the activation and inactivation of these genes are largely unknown. Loss of PTEN function is associated with initiation and progression of EEC[9,10]. Alterations in the number of microsatellite characterise MSI repeats in the DNA of specific cells, such as cancer cells, compared to the DNA of normal cells in the same individual. The instability is due to impairments in the DNA mismatch repair system, resulting in the accumulation of unrepaired errors during DNA replication. To date, very few studies have investigated the prognostic effect of miRNA in EC. Therefore, evaluation of miRNA expression levels may help predict the prognosis of patients with EC. Elucidating the complex interplay between miRNAs, PTEN, and MSI in ECs may reveal key molecular pathways involved in carcinogenesis. Recent data in the literature have suggested that there may be several molecular subtypes of EC, which may have different gene mutations, such as PTEN, KRAS, β-catenin or MSI, leading to altered miRNA synthesis.

The present study was undertaken to determine the expression profiles of miRNAs in EEC and to assess the correlations between these profiles and various clinicopathological factors, including tumor stage, tumor size, depth of myometrial invasion, presence or absence of lymphovascular space invasion, tumor grade, and microsatellite instability status. In this study, we performed analyses of 28 miRNA and mRNA expression levels in samples excised from EEC lesions to identify miRNAs and their potential targets, such as PTEN, KRAS, β-catenin, and MSI. The selection of the 28 miRNAs for our study is typically based on several key criteria, which may vary depending on the focus of the study, such as previous research evidence, expression profiles, biological relevance, experimental validation, and predictive targeting. These miRNAs were selected because of their established potential involvement in the disease under study, supported by previous research, and their relevance in genetic regulatory pathways. miRNAs are likely to be critical in the diagnosis, prognostic prediction and treatment of EC. Therefore, progress in understanding the molecular mechanisms of EC will lead to the development of therapeutic strategies.

Suppose it is known whether or not these factors influence future studies. In this case, miRNA is studied in the endometrial biopsy material taken from the patient for preoperative diagnosis to determine the behaviour of the tumour before staging surgery is performed on the patient before surgery, to determine whether it will recur in time and to decide the extent of surgery during the process, as well as to determine adjuvant chemotherapy and types of radiotherapy. It may be an appropriate approach to evaluate molecular features in patients with low-grade EEC who desire fertility.

Materials and methods

Tissue samples

47 EEC tissue specimens were collected from patients undergoing surgical resection in the gynecologic oncology department. The control group consisted of 50 patients who underwent hysterectomy for ovarian cysts ($n=12$), menorrhagia ($n=15$), and uterine fibroids ($n=23$). The final pathology reports were benign. No patients had undergone chemotherapy or radiotherapy before surgery. Tissues were immediately sent for a frozen section for tumor diameter, myometrial invasion, and tumor grade. An intra-operative frozen section is required to make decisions regarding the surgical steps for staging of EC. Pathologists harvested endometrial tissue specimens at the time of surgery, and small pieces of tumor tissues were stored at -80 °C until the analysis. The procedures used in this study were consistent with the ethical standards of the Human Ethics Committee at Gazi University's Faculty of Medicine, Ankara, Turkey and appropriate informed consent was obtained from each patient (Decision date: 22th February, 2021, Decision number: 195). All methods were performed in accordance with the Declaration of Helsinki and relevant guidelines and regulations.

Total RNA extraction and cDNA synthesis

Total RNA from the EEC tissues was extracted using the TRIzol reagent (Invitrogen) and miRNA isolation kit (GeneAll, South Korea) following the instructions of manufacturers, respectively. The purity and concentration of total RNA were measured by spectrophotometry using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). The samples were stored at -20 °C until use. Total RNA was reversely transcribed into cDNA using Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) and A.B.T.™ cDNA Synthesis Kit (Ankara, Turkey), according to the manufacturer's protocols, respectively.

Quantification of MiRNA levels using quantitative Real-Time PCR (qPCR)

28 miRNAs were identified through a comprehensive literature review and database search for regulators of EEC-related genes and pathways, ensuring that each selected miRNA had a strong biological rationale or prior

association with endometrial cancer. The expression profiles of miRNAs associated with EEC were determined in this study.Quantitative RT-PCR (qPCR) analysis was performed using 2X miRqGreen Master Mix Assay (A.B.T., Ankara, Turkey), according to the manufacturer's protocol. The qPCR primers are shown in Table 1. For miRNA amplification, a two-primer system was used: one primer specific to the mature miRNA sequence and a universal primer supplied by the manufacturer. The primers were 5′- GCTTCGGCAGCACATATACTAAAA T-3′ (forward) and 5′- CGCTTCACGAATTTGCGTGTCAT-3′ (reverse) for U6 snRNA. A total of 20 µl of the mixture was prepared; each sample containing 5 µl RNase-free distilled water, 1 µl of the primer pair, 1 µl ROX Dye (20X), 2 µl of cDNA, and 10 µl of the 2X miRqGreen master mix (A.B.T., Ankara, Turkey). All reactions were performed in triplicates in the 7500 Fast RT-PCR system (Applied Biosystems, USA), with cycle threshold (Ct) values determined using the manufacturer's software. The qPCR conditions for the target miRNAs reaction mixtures were 95˚C for 5 min, 40 cycles of 95˚C for 30 s and 60˚C for 1 min, and finally 95˚C for 5 s. U6 small nuclear RNA (snRNA) level was used as an internal control for the starting amount of cDNA. U6 snRNA (RNU6B) served as an internal control. The fold-change for each miRNA, relative to RNU6B, was calculated using the 2-ΔΔCt method[11,12].

Quantification of mRNA levels using qPCR

To test the expression of a target gene in EEC tissues, qPCR reactions were carried out using the LightCycler® 480 Instrument (Roche Diagnostics, Mannheim, Germany). qPCR analysis was carried out using LightCycler® 480 Probes Master (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. The gene-specific primer sequences and UPL numbers are detailed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an endogenous control to normalize gene expression data. A volume of 2 µL of cDNA product was subjected to real-time PCR in a 10 µL total reaction mixture containing 2.5 µL of LightCycler® 480 Probes Master (Roche Diagnostics, Mannheim, Germany), 0.5 µL forward and reverse primers, 0.1 µL probe and template cDNA. The following thermal cycling conditions were as follows: denaturation at 95 ˚C for 10 min, 45 cycles of amplification: 95 ˚C for 10 s, 60 ˚C for 20 s, and cooling at 40 ˚C for 30 s. After the detection of the Ct values in each sample, expression levels of target genes were calculated using the $2^{-\Delta\Delta CT}$ method[11,12]. Analysis of relative mRNA expression was performed using the comparative Ct with GAPDH as an endogenous control. Each experiment was conducted three times.

miRNA gene	Forward primer (5'- 3')
hsa-miR-let7a	TGAGGTAGTAGGTTGTATAGTT
hsa-miR-18a-3p	ACTGCCCTAAGTGCTCCTTCTGG
hsa-miR21	TAGCTTATCAGACTGATGTTGA
hsa-miR-30b	TGTAAACATCCTACACTCAGCT
hsa-miR-96	TTTGGCACTAGCACATTTTTGCT
hsa-miR-130a	GCTCTTTTCACATTGTGCTACT
hsa-miR-141	CATCTTCCAGTACAGTGTTGGA
hsa-miR-181a	AACATTCAACGCTGTCGGTGAGT
hsa-mirR-182	TTTGGCAATGGTAGAACTCACACT
hsa-miR-183	TATGGCACTGGTAGAATTCACT
hsa-miR-200a	CATCTTACCGGACAGTGCTGGA
hsa-miR-200b	CATCTTACTGGGCAGCATTGGA
hsa-miR-200c	CGTCTTACCCAGCAGTGTTTGG
hsa-miR-203	AGTGGTTCTTAACAGTTCAACAGTT
hsa-miR-205	TCCTTCATTCCACCGGAGTCTG
hsa-miR-429	TAATACTGTCTGGTAAAACCGT
hsa-miR-let7c	TGAGGTAGTAGGTTGTATAGTT
hsa-miR-let7e	ACTCCATCCTCCAACATATCAA
hsa-miR-30c	TGAGGATGAACACTTGTGTGC
hsa-miR-101	CAGTTATCACAGTGCTGATGCT
hsa-miR-125b	ACTGATAAATCCCTGAGACCCTAAC
hsa-miR-126	CATTATTACTTTTGGTACGCG
hsa-miR-129-2	AAGCCCTTACCCCAAAAAGCAT
hsa-miR-217	TACTGCATCAGGAACTGATTGGA
hsa-miR-324-3p	CCCACTGCCCCAGGTGCTGCTGG
hsa-miR-518b	CAAAGCGCTCCCCTTTAGAGGT
hsa-miR-543	AAACATTCGCGGTGCACTTCTT
hsa-miR-596	AAGCCTGCCCGGCTCCTCGGG

Table 1. MiRNA gene specific primer sequences used in qPCR. *qPCR* quantitative real time polymerase chain reaction.

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')	UPL probe number
PTEN	CGAACTGGTGTAATGATATGTGC	CGCCTCTGACTGGGAATAGT	60
KRAS	GGTGGAGTATTTGATAGTGTATTAACC	GAATGGTCCTGCACCAGTAA	62
β-CATENIN	TGTTAAATTCTTGGCTATTACGACA	CCACCACTAGCCAGTATGATGA	8
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	60

Table 2. Gene specific primer sequences and probe numbers. *qPCR* quantitative real time polymerase chain reaction, *UPL* universal probe library.

Interpretation of the morphology

Each tissue specimen was an archive tissue block that was formalin-fixed in a 10% solution, paraffin-embedded. Archived H&E slides were reviewed and selected for the research by a dedicated pathologist; these selected tissues were subsequently re-evaluated for adequacy prior to immunohistochemical processing.

İmmunohistochemistry

Following sectioning at a thickness of 4 µm, the paraffin-embedded blocks were deparaffinized in xylene and then loaded into a Ventana-XT automated staining device (Tucson, AZ).

The antibodies used were monoclonal rabbit anti-human against PTEN (clone: SP218, Ready to use [RTU], Ventana, Tucson, US), monoclonal mouse anti-human against MLH-1 (clone: M1, RTU, Ventana, US), monoclonal rabbit anti-human against MSH-6 (clone: SP93, RTU, Ventana, US), monoclonal mouse anti-human against MSH-2 (clone: G219-1129, RTU, Ventana, US), and monoclonal mouse anti-human against PMS-2 (clone: A16-4, RTU, Ventana, US). Positive controls included the biopsies of endometrium tissue.

Scoring of markers

The immunohistochemical staining was independently assessed by two authors who were unaware of the clinicopathological data. The nuclear immunoreactivity was independently noted at the malignant endometrial epithelium as loss or retained in the optimal fixated area of the block. One case had a heterogeneous expression of PTEN and was included in the loss group.

Statistical analysis

The Relative Expression Software Tool was used, with the Pfaffl method, to calculate relative expression levels of target genes and miRNAs. Differences were assessed with t-tests or one-way ANOVA, and $P < 0.05$ indicated statistically significance.

Results

This study was comprised of 47 patients diagnosed with EEC. The mean age of diagnosis of the patients was 60, (min-max:33–84). Patients were staged according to the 2009 FIGO (International Federation of Gynecology and Obstetrics) criteria. Post-operative grades of the samples diagnosed as EEC were: 16 (34%) grade 1,26 (55.3%) grade 2, and 5 (10.6%) grade 3. Most EEC patients were postmenopausal ($n = 41$). We analyzed 28 miRNAs expression profiles of 47 EEC and 50 normal control. We verified 16 up-regulated and 12 down-regulated miRNAs in the different grades of EEC. Additionally, PTEN, KRAS, and β-CATENİN protein expressions were examined in different grades of EEC.

The levels of MiRNA expression

A total of 28 miRNAs statistically significant differential expression levels between NE and EEC ($p < 0.05$). miRNAs (miR-let-7a, miR-18a-3p, miR-21, miR-30b, miR-96, miR-130a, miR-141, miR-181b miR-182, miR-183, miR-2001, miR-200b, miR-200c, miR- 203, miR-205, and miR-429) had an increased expression in samples from the EEC group compared with samples from the NE group (Fig. 1A). Conversely (miR-let-7c, miR-let-7e, miR-30c, miR-101, miR-125b, miR-126, miR-129-2, miR-217,miR-324-3p, miR-518b, miR-543, and miR-596) were significantly downregulated in the cancerous tissue compared with the NE group (Fig. 1B).

As tumor grade increases, sixteen miRNAs (miR-let-7a, miR-18a-3p, miR-21, miR-30b, miR-96, miR-130a, miR-141, miR-181b miR-182, miR-183, miR-2001, miR-200b, miR-200c, miR- 203, miR-205, and miR-429) were upregulated ($p < 0.05$, $p < 0.01$, ($p < 0.001$; Fig. 2A). On the contrary, twelve miRNAs, including miR-let-7c, miR-let-7e, miR-30c, miR-101, miR-125b, miR-126, miR-129-2, miR-217,miR-324-3p, miR-518b, miR-543, and miR-596, were downregulated($p < 0.05$, $p < 0.01$, ($p < 0.001$; Fig. 2B).

The mRNA levels of PTEN, KRAS and β-CATENIN genes

The mRNA levels of PTEN, KRAS and β-CATENIN genes were evaluated by qPCR. The expression of PTEN, KRAS and β-CATENIN mRNA levels were markedly decreased in EEC groups compared to NE group ($p < 0.05$; Fig. 3A).

The expression of KRAS and β-CATENIN mRNA levels was decreased in Grade 2 group when compared with NE group, no statistically significant changes were observed ($p > 0.05$). Furthermore, PTEN mRNA levels were also markedly decreased in Grade 2 group compared to NE group ($p < 0.05$; Fig. 3B). Additionally, the levels of KRAS and β-CATENIN mRNA were significantly decreased in Grade 3 group compared to the NE group

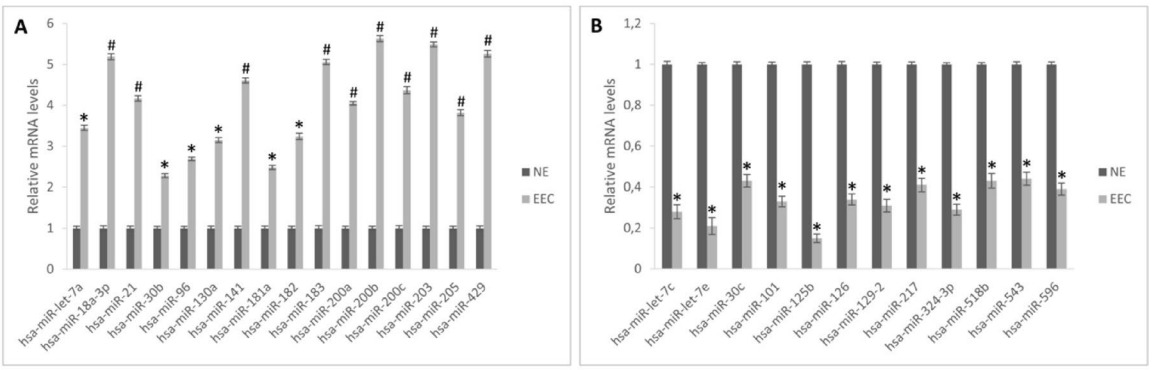


Fig. 1. **(A)** Relative expression levels of increased miRNAs in NE and EEC tissues. **(B)** Relative expression levels of decreased miRNAs in NE and EEC tissues. $^{*}p < 0.05$, $\#p < 0.01$ versus control cells. EEC: Endometrioid type Endometrial Cancer; NE: Normal Endometrium qPCR: Quantitative real-time polymerase chain reaction; RNU6B: U6 small nuclear 2.

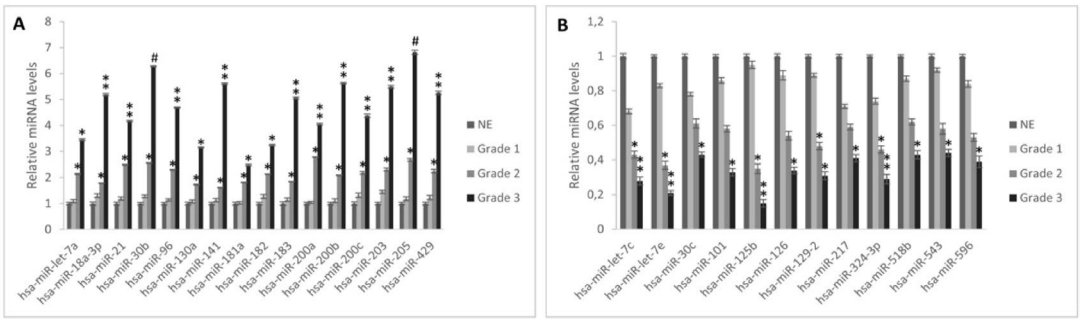


Fig. 2. **(A)** Relative expression levels of increased miRNAs in NE and EEC tissues with different tumor grade. **(B)** Relative expression levels of decreased miRNAs in NE and EEC tissues with different tumor grade. $^{*}p < 0.05$, $^{**}p < 0.01$, $\#p < 0.001$ versus NE group. EEC: Endometrioid type Endometrial Cancer; NE: Normal Endometrium; qPCR: Quantitative real time polymerase chain reaction; RNU6B: U6 small nuclear 2.

($p < 0.05$). Moreover, PTEN mRNA levels were also markedly decreased in Grade 3 group compared to NE group ($p < 0.01$; Fig. 3B).

MMR, PTEN protein expression of EEC and NE

Deficient mismatch repair (dMMR) was detected in 13 of 47 (28%). Loss of PTEN expression was detected in 31 of 47 (66%). Co-deficiency of mismatch proteins and PTEN was in 9 of 47 (13%). Loss of MSH2 and MSH6 was in 1 of 13 (8%) while loss of PMS2 and MLH1 was seen in the rest dMMR (92%). Expression loss of immunohistochemical markers were usually diffuse but 1 biopsy had heterogeneous PTEN and was accepted as deficient (Fig. 4).

Positive, heterogenous and negative immunohistochemical expressions are represented in Figs. 5 and 6.

When re-sorted with FIGO grades; 4 of 16 (25%) grade 1 were dMMR and 10 (63%) were PTEN deficient. 2 (13%) were co-deficient. 5 of 26 (19%) grade 2 were dMMR and 17 (65%) were PTEN deficient. 4 (15%) were co-deficient. 4 of 5 (80%) grade 3 were dMMR and PTEN co-deficient while 1 was pMMR and PTEN retained (Table 3).

Discussion

Cancer is a complex pathological condition resulting from the interaction of genetic, molecular, and environmental factors. Understanding their precise roles and interactions within the context of EC is challenging due to the multifaceted nature of cancer mechanisms. Currently, the diagnosis of EC is primarily based on histopathological examination of tissue samples taken during surgery. This classification then guides the choice of treatment for the patient. However, the use of histopathology alone for prognosis has limitations. Determining the extent of surgical treatment, such as whether to include lymph nodes during hysterectomy, remains a challenge. Factors such as lymph node status, lymphovascular space invasion, and histological type provide information, but they may not be comprehensive enough to guide precise treatment decisions. miRNA may serve as an additional prognostic factor in EC, and may improve histopathological diagnosis. Studying miRNA alongside histology and frozen pathology results could potentially improve the accuracy of surgical planning. This could potentially lead

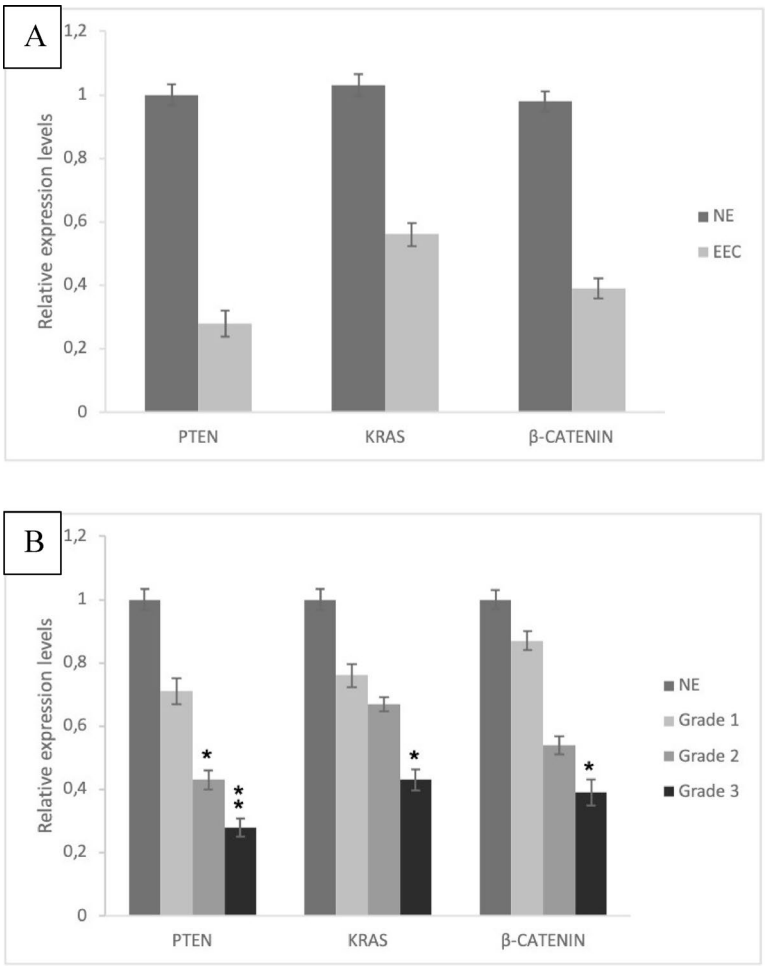


Fig. 3. **(A)** Relative expression levels of PTEN, KRAS, and β-CATENIN mRNAs in NE and EEC tissues. **(B)** Relative expression levels of PTEN, KRAS and β-CATENIN mRNAs in NE and EEC tissues with different tumor grade. $^{*}p < 0.05$, # $p < 0.01$ versus control cells. EEC: Endometrioid type Endometrial Cancer; NE: Normal Endometrium; qPCR: Quantitative real-time polymerase chain reaction; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

to more targeted and effective surgical interventions. Beyond its role in surgical guidance, miRNA analysis could also have wider implications. Examining miRNA in urine and serum samples from high-risk patients could provide a screening method for EC in the future. In light of this information, we performed analyses of miRNA and mRNA expression levels in samples excised from EEC lesions to identify 28 miRNAs and their possible targets, such as PTEN, and MSI. We identified 16 miRNAs with increased expression levels and 12 miRNAs with decreased expression levels in EEC tissues. We also found that PTEN protein and mRNA levels and KRAS, and β-catenin mRNA levels were decreased in EEC tumor tissues compared with non-tumor tissues. The study's results revealed a significant correlation between miRNAs' expression levels and the target genes' mRNA levels (PTEN, KRAS, and β-Catenin) in the tumor tissues.

Cell growth, proliferation, signalling, and other cellular processes are regulated by the essential genes PTEN, KRAS, and β-catenin. Regulation of cell growth and division is a critical function of the tumor suppressor gene PTEN. PTEN inactivation is frequently observed in various cancers, contributing to uncontrolled cell proliferation and enhanced tumor development. MiRNAs may play a role in this inactivation by targeting the PTEN for degradation or translational inhibition. Suppression of PTEN by miRNA interactions may influence the response of cancer cells to chemotherapy. Identifying the specific miRNAs that interact with PTEN provides insight into the molecular mechanisms responsible for PTEN inactivation. If specific miRNAs are consistently associated with PTEN suppression in a specific cancer type, targeting these miRNAs could be explored as a therapeutic option to restore PTEN function and improve treatment response. PTEN has been confirmed as a target gene of miR-21, miR-181a, miR-182, miR-183, miR-200a, miR-200c, and miR-205 in a variety of malignancies especially EC[13–19]. A previous study showed that EC tissues had significantly higher expression of miR-182, 183, 200a, 200c, and 205 compared to NE tissues. Immunohistochemical analysis of the same endometrial tissues showed a loss of PTEN expression. Significantly higher miR-200c expression was observed in PTEN-negative tissues compared to PTEN-positive tissues[14].The downregulation of miR-205, one of these miRNAs, and its targeting of the PTEN gene suggests a potential mechanism by which miR-205 may

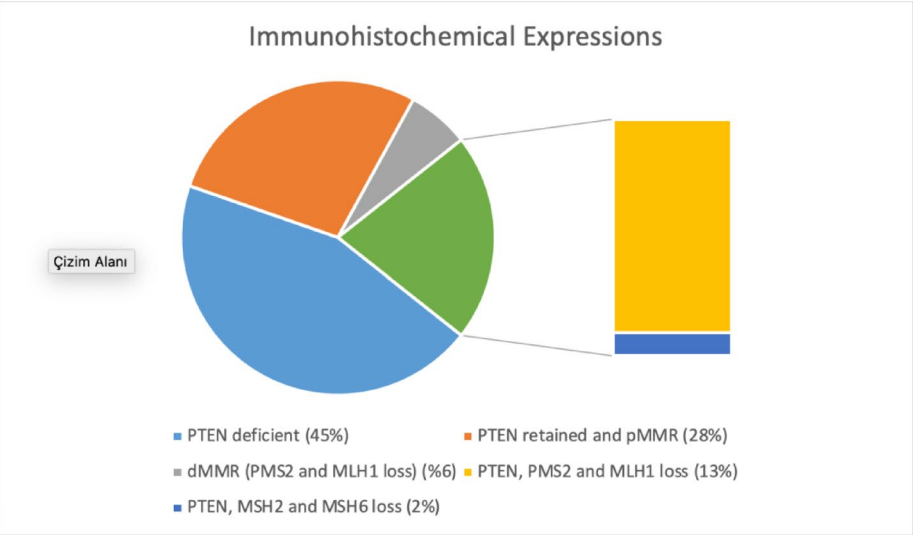


Fig. 4. Immunohistochemical expression rates. dMMR: Mismatch repair deficient, pMMR: Mismatch repair proficient, PTEN: Phosphatase and tensin homolog protein, PMS2: Mismatch repair endonuclease PMS2, MLH1: DNA mismatch repair protein Mlh1, MSH2: DNA mismatch repair protein Msh2, MSH6: DNA mismatch repair protein Msh6.

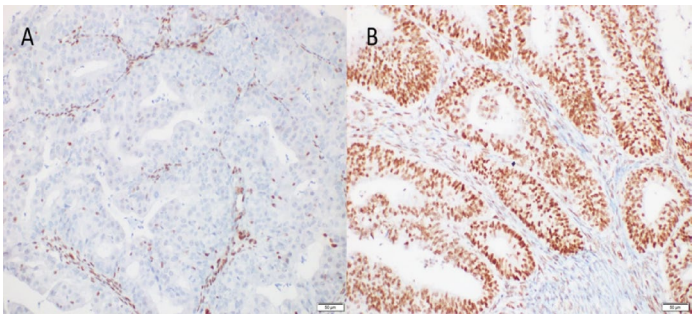


Fig. 5. Micrographs representing an example of the immunohistochemical results of mismatch repair proteins. (A) MLH1 is positive in the endothelial, stromal and inflammatory cells surrounding the tumor islands while the tumor nuclei are negative / deficient. (*x200 magnification*) (B) A tumor with proficient mismatch repair proteins with positivity in both tumor cell nuclei and background stroma. (*x200 magnification*)

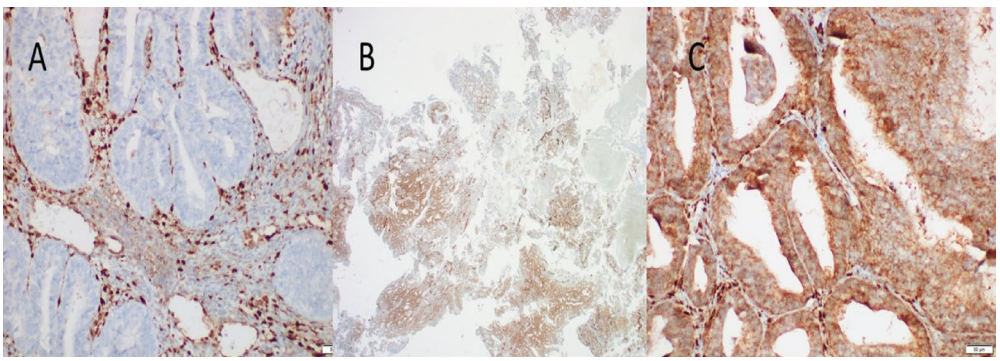


Fig. 6. Micrographs representing the immunohistochemical results of phosphatase and tensin homolog protein. (**A**) PTEN is positive in the endothelial, stromal and inflammatory cells surrounding the tumor islands and the tumor nuclei and cytoplasm are negative / deficient. (*x200 magnification*) (**B**) The only tumor with heterogonous expression with both positive and negative tumor areas (*x12,5 magnification*) (**C**) A tumor with proficient PTEN with positivity in both tumor and background stromal cells. (*x200 magnification*)

FIGO Grade	Total	Co-deficient MMR and PTEN	dMMR	PTEN deficient	pMMR	PTEN retained
1	16	2	4	10	12	6
2	26	4	5	17	21	9
3	5	4	4	4	1	1

Table 3. Distribution of dMMR and PTEN deficiency across FIGO grades in endometrial Cancer. dMMR: Mismatch repair deficient, pMMR: Mismatch repair proficient, FIGO: Federation of Gynecology and Obstetrics, PTEN: Phosphatase and tensin homolog protein.

contribute to decreased survival in certain cancers. Reduced levels of PTEN due to miR-205 targeting could lead to uncontrolled cell growth and survival, which could affect patient outcomes[15,16]. In another study, miR-205 targeting of PTEN was found to be significantly associated with reduced survival in EC, suggesting miR-205 as a potential prognostic biomarker[17]. Furthermore, miR-205 directly regulates PTEN expression in EC cells, inhibiting cellular apoptosis. One study, an inverse correlation was noted between PTEN protein expression and increased miR-21 in EEC. Similarly, our study found significantly increased expression of miR-21, miR-181a, miR-182, miR-183, miR-200a, miR-200b, miR-200c, and miR-205, along with markedly decreased PTEN mRNA levels, in EEC and Grade 3 groups compared to the NE group. A study by Geletina et al. demonstrated significantly increased levels of miR-181a in a mixed cohort of endometrial cancer tissues compared to normal endometrium. This study also indicated that high miR-181a expression correlated negatively with PTEN levels in tumors from non-obese individuals with EC[19]. Other research has associated miR-181a overexpression with enhanced tumor growth, cell migration, invasiveness, and metastatic potential[20–22].

As a major component of the Wnt pathway, β-catenin is central to the transduction of Wnt signals. It plays a critical role in various aspects of cancer progression, including tumourigenesis, angiogenesis and metastasis of cancer cells. miRNAs are known to regulate the expression of components within the Wnt/β-catenin signalling pathway[23–26]. Understanding how miRNAs affect the Wnt/β-catenin pathway may provide insights into potential therapeutic strategies. he miR-200 family members have been shown to reduce cancer cell motility by suppressing WNT/β-catenin signalling. The miR-200 family consists of five members: miR-200a, miR-200b, miR-200c, miR-141, and miR-429[24,25]. The tumour suppressor role of miR-200a and its direct regulation of β-catenin have been confirmed in gastric adenocarcinoma and hepatocellular carcinoma. miR-200b has also been shown to reduce the growth of Wnt-1 in gastric cancer[26]. Consistent with these findings, in our study, miR-200a, miR-200b, miR-200c, miR-141 expression levels were also significantly increased and KRAS mRNA levels were markedly decreased in EEC groups and grade 2 and 3 groups compared to the NE group.

Epithelial-to-mesenchymal transition (EMT), a critical factor in tumorigenesis, is thought to be influenced by the Wnt/β-catenin signalling pathway. Studies have shown that miR-543 activates the Wnt/β-catenin pathway[27,28]. Compared to NE tissue, malignant endometrial tissue has decreased endogenous miR-543 expression. Furthermore, miR-543 has been shown to suppress the proliferation, migration, and invasion of EC cells[29]. Conversely, overexpression of miR-543 in endometrial epithelial and adenocarcinoma cells reduces their migratory, proliferative, and invasive abilities, while significantly increasing apoptosis[30]. In the current study, we found that miR-543 inhibits β-catenin mRNA expression.

Cell growth and differentiation are regulated by the crucial gene KRAS. Mutations in KRAS can lead to uncontrolled cell proliferation and promote cancer development. Dysregulation of miRNA expression can affect KRAS protein levels and its activity, further influencing cancer development and progression. In cancers with a KRAS mutation, miR-324–3p and miR-518b miRNAs are downregulated, suggesting that they may have a tumor-suppressive role or be involved in regulating pathways associated with cancer development. The downregulation of these miRNAs could potentially contribute to the activation of KRAS-driven pathways and disease progression[31]. Another study reported that miR-18a-3p, which is upregulated in EC, acts as a tumor suppressor by targeting KRAS[32]. Given the previously documented differential expression of miR-200c in breast tumor tissues, an investigation was undertaken to assess the expression levels of miR-200c and KRAS protein in a panel of breast cancer cell lines. The study showed an inverse correlation between miR-200c and KRAS protein expression; specifically, breast cancer cells exhibiting with high levels of miR-200c had correspondingly low levels of KRAS protein[33]. Tanaka et al. studied 224 human pancreatic neoplasms and found that EVI1 regulates KRAS protein levels and the KRAS-ERK pathway through transcriptional control of miR-96 and miR-181. Their research showed that introducing miR-96 (but not miR-181) into cells reduced KRAS protein expression and caused cell cycle arrest, suggesting a tumour suppressor role for miR-96 in EVI1-mediated KRAS regulation[34]. Liao et al. extended their studies to miR-30b, which is known to be downregulated expression in colorectal cancer. miR-30b affects tumor growth by targeting multiple genes, including KRAS[35]. Consistent with these findings, in our study, the expression levels of miR-18a-3p, miR-30b, miR-200c, miR-96, and miR-181 were also significantly increased and KRAS mRNA levels were markedly decreased in the EEC groups and grade 2 and 3 groups compared to the NE group.

MSI is defined as the accumulation of mutations within microsatellites, which are short, repetitive regions of DNA. This instability is a consequence of impairments in the DNA mismatch repair (MMR) system, the cellular machinery that normally corrects errors that occur during DNA replication. The interplay between miRNAs and deficient MSI in EC is poorly characterised and complex. Dysregulation of miRNAs, assuming they play a role in controlling the expression of DNA repair and MMR genes, could potentially be a factor in the initiation or progression of MSI-deficient EC. Many miRNAs have been studied in cancer, but their specific

relationships with MSI and EC cannot be fully characterised. Among them, miR-21 miRNA is commonly upregulated in various cancers and promotes cell proliferation, inhibits apoptosis, and enhances invasion and metastasis[36,37]. miR-21 expression was also found to be increased in EEC compared to normal tissue in our study and increased with increasing tumor grade, whereas miR-21 expression was found to be decreased in EEC in the study by Tsukamoto et al.[16]. On the one hand, the miR-200 family (miR-200a, miR-200b, miR-200c) was found to have increased expression when healthy tissue was compared to tumor tissue in our study and by Liu and Hermyt et al.[25,38]. Decreased expression of miR-203 is a common feature of cancer, and this microRNA exerts tumorsuppressive effects by targeting genes involved in the processes of cell proliferation and invasion. Its relationship with MSI is unclear but could influence the overall tumor phenotype. In our study, the expression of miR-200a, miR-200b, miR-200c, miR-203 expression increased in EEC compared to normal tissue with increasing tumour grade. Another study reported a high incidence of miR-203 hypermethylation in endometrial cancer (EC), which closely correlated with MSI and the methylation status of MLH1. The methylation status of miR-203 may therefore be a predictive biomarker for individuals diagnosed with endometrioid and clear cell carcinoma[39–41]. miR-129-2 also contributes to MSI and hMLH1 methylation, the latter being a DNA mismatch repair gene involved in type I EC development[42]. In our study, miR-129-2 expression levels decreased in EEC compared to normal tissue with increasing tumour grade.

Some miRNAs, such as miR-30c, miR-101, miR-125b, miR-217, and miR-596 may have implications in cancer, but their specific relationships with PTEN, KRAS, β-catenin, and MSI in EEC have not been widely reported. In our study, miR-181a expression levels were significantly increased, whereas miR-30c, miR-101, miR-125b, miR-129-2, miR-217, miR-543, and miR-596 were markedly decreased in the EEC group and the grade 3 group when compared with the NE group.

EC exhibits reduced miR-30c expression, as demonstrated in previous studies[43,44]. miR-30c has tumor suppressor activity and suppresses EC cell growth by targeting MTA1. One of these studies also showed that while miR-30c is downregulated in EC tissues, but is highly expressed in ER-negative HEC-1-B cells[43]. miR-30c expression levels were also significantly reduced in the EEC groups and grade 3 group compared to the NE group in our study. In a study by Konno et al., miR-101 levels were significantly decreased in aggressive endometrial cancer cell lines compared to immortalised human endometrial epithelial cells[44–46]. In another study, Liu et al. found that miR-101 levels were significantly reduced in EC tissue was significantly reduced compared to that of adjacent normal tissue[47]. Consistent with these findings, we also found that miR-101 levels were significantly downregulated in the EEC groups and grade 2 and 3 groups compared to the NE group. miR-125b is a small non-coding RNA that is important for controlling gene expression. It is known to be ubiquitously expressed, meaning that it is found in many different tissues and cell types. However, its expression levels can vary in different types of tumors, and these variations lead to the development and progression of cancer. In contrast, other cancers, including breast cancer and hepatocellular carcinoma, show significantly reduced miR-125b expression. This term "downregulation" denotes a decrease in the cellular level of the miRNA.This means that the normal checks and balances that regulate cell growth and prevent the survival of damaged cells are disrupted, contributing to the malignant transformation of cells and the development of cancer. Aberrant miR-125b expression has diverse cellular effects that vary depending on the context and interactions with other pathways in different cancers[48]. Compatible with these findings, we also found that miR-125b levels were significantly reduced in the EEC groups and grade 2 and 3 groups compared to the NE group.

This study is the first, to the best of our knowledge, to evaluate the correlation between the relative expression of miRNA and both tumor grade and FIGO stage, specifically within EEC tumors. The purpose of the current study is to compile and present an up-to-date summary of how miRNAs are involved in the processes of tumor initiation and progression, as well as how they influence the prognosis of EEC. miRNAs can potentially be non-invasive biomarkers that aid in the diagnosis of nodal status, determine the extent of staging surgery, predict recurrence, and guide decisions regarding adjuvant therapy. These markers can be obtained from readily available sources such as blood or other body fluids, making them convenient tools for early detection and monitoring. One limitation of the present study is the lack of statistical correlation analysis between miRNA and mRNA levels due to limited sample size and statistical power; this is intended to be addressed in future studies with larger cohorts. A better understanding of miRNAs and the regulatory networks of PTEN, β-catenin, KRAS and MSI will provide insights to further the development of miR-based therapeutics.

Data availability

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

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
This study approved by the Ethics Committee of Gazi University Hospital and appropriate informed consent was obtained from each patient (Decision date: 22th February, 2021, Decision number: 195).

Additional information
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