Serum expression levels of HOTAIR and miR-646 in endometrial cancer

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Abstract. Endometrial cancer (EC) is the fourth most common malignancy in women in high-income countries and the sixth globally, with ~417,000 new cases and ~97,370 mortalities reported worldwide in 2020. Its incidence has increased by >130% over the past 30 years, due to advances in FIGO-based staging systems and earlier detection. However effective non-invasive biomarkers for early diagnosis and staging are limited. Non-coding RNAs (ncRNAs), particularly long ncRNAs (lncRNAs) and microRNAs (miRs), are critical regulators in the biology of cancer, and may potentially serve as novel diagnostic and prognostic biomarkers. In the present study, serum levels of the lncRNA Hox antisense intergenic RNA (HOTAIR) and the miR-646 were evaluated in 71 patients with EC and 62 matched healthy controls using reverse transcription-quantitative PCR. The results showed that the serum HOTAIR expression level was significantly increased in patients with EC compared with controls. This suggested that it may have potential as a diagnostic biomarker. Although overall miR-646 levels did not differ significantly between patients with EC and healthy controls, a notable reduction in miR-646 levels were observed in patients with advanced clinical T stages compared with those with early stages. This indicated an association with disease progression. Receiver operating characteristic curve analyses indicated that a serum HOTAIR level of 1.08 may distinguish patients with EC from healthy controls with 63.3% sensitivity and 61.2% specificity. Additionally, a miR-646 cut-off value of 0.655 differentiated patients with advanced-stage from early-stage EC with 72.7% sensitivity and 66.7% specificity. These findings suggested that serum HOTAIR may be a potential diagnostic biomarker and that miR-646 may be a potential indicator of disease progression in EC. However, further validation in larger patient cohorts and investigation of exosomal expression levels are necessary in order to assess the potential clinical relevance and therapeutic potential of HOTAIR and miR-646.

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Introduction

Endometrial cancer (EC) is one of the most common malignancies in women, ranking fourth in high-income countries and sixth globally, with ~417,000 new cases diagnosed worldwide in 2020. Its incidence has increased by >130% over the past three decades, with ~97,370 mortalities reported worldwide in 2020 (1). Risk factors for EC include obesity, a high glycaemic index diet, early menarche, late menopause, advanced age, diabetes, the use of oestrogen, nulliparity, polycystic ovary syndrome and a sedentary lifestyle (2-4). Prolonged exposure to unopposed oestrogen, particularly through hormone replacement therapy without concomitant progestin, increases the risk of developing EC by 4.5-8.0-fold (2). The pathogenesis of EC is categorised into two primary subtypes in a study by Bokhman (5). Type I is associated with oestrogen and offers a more favourable prognosis, whereas type II represents a high-grade variant with a poorer prognosis, typically observed in older individuals (aged 55-60 years) (6).

According to the Human Genome Project, >90% of the genome is considered to be transcribed (7). Research indicates that only 2% of these synthesised transcripts can encode proteins, while >75% consists of non-protein-coding RNAs (8). Non-coding RNAs (ncRNAs) are classified into two categories based on their size, namely long ncRNAs (lncRNAs) and small ncRNAs. These categories can be further divided based on size, cellular localisation or function (9). In particular, lncRNAs and microRNAs (miRs) serve crucial roles in epigenetic regulation, control of gene expression levels and the regulation of cellular processes including proliferation, apoptosis, migration, invasion and the epithelial-mesenchymal transition (9-13). Previous studies demonstrate an association between the expression of ncRNA and various pathological conditions, including neurological, cardiological and cancer-related diseases (12-14).

lncRNAs are generally longer than 200 nucleotides. They are considered to contribute to carcinogenesis by binding to transcription factors or repressors, acting as signalling molecules to activate or inhibit the expression of genes and engaging with protein complexes and cofactors (13). Moreover, lncRNAs also serve a role in post-transcriptional regulation by participating in mRNA processing. Additionally, lncRNAs function as sponges, preventing miR from binding to their target mRNA (15).

The study by Rinn *et al* (14) was the first to report and characterize Hox antisense intergenic RNA (HOTAIR) as a lncRNA. Studies demonstrate that HOTAIR helps regulate gene activity, which has led to investigations on its potential connection to various types of cancer, including lung, gastric, glioma, pancreatic, cervical (16-20) and EC (21,22). The expression of HOTAIR increases in patients with EC, and its elevation is associated with advanced disease, metastasis and drug resistance (16,17,22). However, the molecular effects of HOTAIR in EC and the potential clinical application of its serum levels have not been fully elucidated.

miRs are small ncRNA sequences, ~22 nucleotides in length, involved in regulating processes such as development, cell differentiation, apoptosis and proliferation. Their primary function is gene silencing (23). miRs can act as either tumour suppressors or oncogenes under specific conditions (24). While oncogenic miRs enhance the tumour cell phenotype, tumour suppressor miRs diminish it (25). The abundant presence of miRs in faeces, sputum, pleural effusion and urine suggests that alterations in miR expression levels are readily detectable.

Previous studies indicate that miR-646 is linked to various types of cancer, including lung (26), laryngeal (27), clear cell carcinoma (28), retinoblastoma (29) and osteosarcoma (30), with a notable role in gastrointestinal (31) and gynaecological tumours (32). It is hypothesized to function as a tumour suppressor in these malignancies, and in EC, it primarily reduces tumorigenic potential through the downregulation of nucleophosmin 1 (NPM1) (32,33). *In vitro* studies by Zhou *et al* (33) demonstrates that HOTAIR increases NPM1 levels by suppressing the expression of miR-646, which promotes cancer cell proliferation, invasion and migration.

At present, there is not a reliable non-invasive biomarker that is routinely used for the diagnosis or monitoring of EC. HOTAIR is a potential biomarker candidate due to its overexpression and association with advanced stages of the disease (33). Additionally, miR-646 may also serve as a detectable marker in body fluids (34). Experimental evidence from Zhou *et al* (33) suggests a possible regulatory interaction between HOTAIR and miR-646; however, this association is yet to be confirmed in clinical serum samples. Therefore, the present study aimed to investigate the serum expression levels of HOTAIR and miR-646 in patients with EC and investigated their potential as non-invasive diagnostic or prognostic biomarkers.

Materials and methods

Subjects. The study population consisted of women aged ≥18 years with histologically confirmed EC, diagnosed by endometrial biopsy and managed surgically. All participants provided written informed consent prior to inclusion. Patients with biopsy-proven EC of any histopathological subtype who underwent hysterectomy with surgical staging and subsequent histopathological evaluation of the surgical specimens were included. The control group included women who attended the general gynaecology outpatient clinic for non-malignant gynaecological conditions and who had no history of malignancy. Patients were excluded if they declined to provide informed consent, were medically inoperable, refused surgical treatment, had a final pathology not confirming EC, had synchronous or other primary malignancies, had recurrent disease, or had received prior oncologic treatments such as chemotherapy, radiotherapy or immunotherapy. The present study examined the association between HOTAIR and miR-646 gene expression levels and the development of EC. Additionally, the association between prognostic factors of EC, such as grade and clinical T stage, and the expression of HOTAIR and miR-646 was investigated. In total, 71 patients that were admitted to the Department of Obstetrics, Gynaecology-Oncology Division, Istanbul Medical Faculty, Istanbul University (Istanbul, Turkey) and diagnosed with EC based on biopsy results, were included in the present study. Additionally, 62 healthy women who sought services at the Department of Obstetrics and Gynaecology, Istanbul Medical Faculty, Istanbul University, for routine gynaecological examination or benign conditions were included in the control group. Patients and healthy control individuals were enrolled in the present study between August 2021 and March 2023. The study protocol was reviewed and approved by the Institutional Review Board and Ethics Committee of Istanbul University (Istanbul, Turkey; approval no. 272576; approval date, 01.07.2021). Sample collection and experimental analyses were completed by June 2023. Blood samples were collected after both the patients and control individuals were informed verbally and in writing.

Patients and controls were matched based on age, body mass index (BMI) and smoking status. An individual was classed as a smoker if they had a minimum of 10 packs/year. An individual was classed as a non-smoker if they had either quit smoking more than a year before the present study or had never smoked. All participants in both groups were female. The median ages were 57 years (range, 29-81 years) in the patient group and 54 years (range, 34-77 years) in the control group. The mean age ± standard deviation (SD) was 57.7±11.0 years for the patient group and 54.8±10.1 years for the control group. Tumour stage and grade were determined according to surgical staging. In the present study, EC was classified as low grade [International Federation of Gynecology and Obstetrics (FIGO) grades 1-2 endometrioid)] and high grade (FIGO grade 3 endometrioid and non-endometrioid types), which was consistent with accepted dichotomy in the literature (35). Previous studies define high-grade EC to include grade 3 endometrioid tumours, serous, clear cell, undifferentiated and carcinosarcoma subtypes (6,36,37). These tumours are characterized by more aggressive biological behaviour, a poorer prognosis compared with low-grade (FIGO grade 1-2 endometrioid) endometrial carcinomas and as high risk by major clinical guidelines, including the European Society of Gynaecological Oncology, the European Society for Radiotherapy and Oncology, and the European Society of Pathology guidelines for EC (2021) (38) and the National

Comprehensive Cancer Network Clinical Practice Guidelines in Oncology: Uterine Neoplasms (version 3.2025) (39). Stages IA and IB were regarded as early stages, while stages II-IV were considered advanced stages. Additionally, cancer antigen 125 (CA125) levels, menopausal status, hypertension, diabetes mellitus, recurrence, lymph node involvement, disease-free survival, overall survival and cancer-related mortality were evaluated.

Reverse transcription-quantitative PCR (RT-qPCR). For RNA extraction, venous blood samples were allowed to clot for 30 min, and were then centrifuged at 1,500 x g for 10 min at 4˚C. The separated serum samples were stored at -80˚C until further analysis. RNA isolation was carried out using the miRNeasy Serum/Plasma Advanced kit (cat. no. 217204; Qiagen GmbH). The concentration and purity of the extracted RNA samples were evaluated using an Eon Biotek instrument. Following measurement, RNA samples were stored at -80˚C. For miRNA expression level analysis, reverse transcription was carried out using the miRCURY LNA RT kit (cat. no. 339340; Qiagen GmbH) according to the manufacturer's protocol. The reaction was carried out at 42˚C for 1 h, followed by enzyme inactivation at 95˚C for 5 min. For lncRNA expression level analysis, cDNA synthesis was conducted using the RT^2 PreAMP cDNA Synthesis kit (cat. no. 330451; Qiagen GmbH) following the manufacturer's protocol. The reverse transcription step was carried out at 37˚C for 1 h, followed by enzyme inactivation at 95˚C for 5 min. RT-qPCR was carried out on a Rotor-Gene Q Real-Time PCR System (Qiagen GmbH) using the miRCURY LNA SYBR Green PCR kit (cat. no. 339345; Qiagen GmbH) for the detection of hsa-miR-646, and the RT^2 SYBR Green ROX FAST Mastermix kit (cat. no. 330620; Qiagen GmbH) for the analysis of lncRNA HOTAIR expression levels. PCR amplification was carried out according to the manufacturer's protocols. For lncRNA, initial denaturation was carried out at 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 1 min. For miR, initial denaturation was carried out at 95˚C for 2 min, followed by 40 cycles of 95˚C for 10 sec and 56˚C for 1 min.

The primers used in the present study were obtained from Qiagen GmbH. They included the following: RT^2 qPCR Assay for GAPDH (cat. no. PPH00150F), RT^2 lncRNA qPCR Assay for HOTAIR (cat. no. LPH07360A), miRCURY LNA miRNA PCR Assay for hsa-miR-646 (cat. no. YP00204546) and miRCURY LNA™ miRNA PCR Assay for hsa-U6 (cat. no. YP02119464). The exact sequences of these primers are proprietary and not publicly available from the manufacturer. Gene expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (40). Relative expression levels were normalized to GAPDH for lncRNA and to U6 for miR, in accordance with previously published studies (22,27,41-43).

Statistical analysis. SPSS version 21 (IBM Corp.) was used for data analysis. The distribution of data in the present study was assessed using the Shapiro-Wilk test. Variables with normal distribution were analysed using one-way ANOVA followed by the Bonferroni post hoc test, while non-normally distributed variables were analysed using the Mann-Whitney U test. In the present data, 'age' had a normal distribution, whereas 'BMI' did not follow a normal distribution. The Chi-square test was used to analyse the association between qualitative data. Spearman's correlation test was used to investigate the association between two datasets. Receiver operating characteristic (ROC) curve analysis was carried out to determine whether an independent variable could distinguish between cases of EC and healthy controls. The ROC curve was used to identify the optimal predictive value and to calculate the sensitivity and specificity of the predictive value. The Kaplan-Meier method was used to compare survival between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

There were no significant differences between the control group and patients with EC in terms of age, BMI, smoking, hypertension or diabetes mellitus status. Among the patients, 46 cases (64.8%) had low-grade tumours (grades 1 and 2), while 25 cases (35.2%) had high-grade tumours (grade 3), of which 2 cases (8%) were of non-endometrioid histology (1 case had serous carcinoma and another case had clear cell carcinoma). Early-stage disease (stages IA and B) was present in 84.5% of the patients, whereas 15.5% had advanced-stage disease. Tumour recurrence was observed in 14.1% of cases, and lymph node involvement was identified in 87.3% of patients. Mean disease-free survival and overall survival were 30.06±10.42 and 32.18±7.98 months, respectively, while cancer-related mortality was 10.2% (Table I).

The expression of HOTAIR was significantly increased in patients with EC compared with the control group (P=0.020). However, the expression of miR-646 did not differ significantly between the patient and the control groups (P=0.579). Additionally, no significant differences in HOTAIR or miR-646 expression levels were revealed between low- and high-grade tumours (P=0.230 and P=0.377, respectively). The HOTAIR expression levels did not vary significantly between low and high clinical tumour stages (P=0.466). However, the miR-646 expression level was significantly reduced in the high tumour stage group compared with the low tumour stage group (P=0.045). Recurrence status and lymph node involvement showed no significant association with the expression levels of HOTAIR (P=0.321 and P=0.192, respectively) or miR-646 (P=0.741 and P=0.942, respectively) (Fig. 1). Spearman's correlation analysis did not demonstrate a significant association between serum miR-646 and HOTAIR expression levels (Table II). Furthermore, there were no significant correlations between the miR-646 or HOTAIR expression levels and CA125 concentrations (Table III).

ROC analysis was carried out to investigate the optimal cut-off values for serum HOTAIR and miR-646 expression levels to distinguish between patients with EC and the healthy controls. For HOTAIR, an expression level threshold of 1.08 was revealed to differentiate between patients with EC and healthy controls, with a sensitivity of 63.3% and a specificity of 61.2%. Therefore, patients were classified as being HOTAIR-positive (with a value of ≥1.08) or HOTAIR-negative (with a value of <1.08). For miR-646, an expression level cut-off value of 0.655 was demonstrated to distinguish between patients with advanced-stage and early-stage EC, with a sensitivity of 66.7% and a specificity of 72.7%. Therefore, patients were categorized as being miR-646-positive (≥0.655) or miR-646-negative (<0.655). These classification criteria are presented in Fig. 2.

Table I. Comparison of demographic and clinical characteristics between the control and patient groups.

Parameter	Healthy control individuals (n=62)	Patients with endometrial cancer (n=71)	P-value
Age, years (mean ± SD)	54.77±10.09	57.64±11.03	0.118
BMI, kg/m^2 (mean ± SD)	31.20±6.37	32.24±8.36	0.261
CA125, median (min-max)	-	13.0 (5.0-745.0)	
Smoking status, N (%)			0.249
Non-smoker	39 (63.9)	52 (73.2)	
Smoker	22 (36.1)	19 (26.8)	
Menopause status, N (%)			-
Premenopausal	-	24 (33.8)	
Postmenopausal	-	47 (66.2)	
Tumour grade, N (%)			-
Low grade (grades 1 and 2)	-	46 (64.8)	
High grade (grade 3 endometrioid and non-endometrioid histology types)	-	25 (35.2)	
Stage, N (%)			-
Early stage (IA and B)	-	60 (84.5)	
Advanced stage (II-IV)	-	11 (15.5)	
Recurrence, N (%)			-
No	-	61 (85.9)	
Yes	-	10 (14.1)	
Lymph node involvement, N (%)			-
Yes	-	62 (87.3)	
No	-	9 (12.7)	
Hypertension, N (%)			0.070
Yes	13 (21.0)	25 (35.2)	
No	49 (79.0)	46 (64.8)	
Diabetes mellitus, N (%)			0.150
Yes	8 (12.9)	16 (22.5)	
No	54 (87.1)	55 (77.5)	
Disease-free survival, months (mean ± SD)	-	30.06±10.42	-
Overall survival, months (mean ± SD)	-	32.18±7.98	-
Cancer-related mortality, N (%)			-
No	-	53 (89.9)	
Yes	-	6 (10.2)	

BMI, body mass index; CA125, cancer antigen 125; min-max, minimum-maximum.

No statistically significant differences in overall survival (in months) were observed between the HOTAIR-positive and -negative groups or between the miR-646-positive and -negative groups (Fig. 3).

Discussion

Although the incidence rate of EC has increased by >130% over the past three decades, with >417,000 new cases and ~97,370 mortalities reported worldwide in 2020 (1), reliable non-invasive diagnostic biomarkers are still unavailable. Advancing diagnostic methods and therapeutic strategies depends on elucidating the molecular pathways involved in this disease. ncRNAs, including lncRNAs and miRs, have garnered attention as potential biomarkers across various types of cancer such as breast, gastric and gynaecological cancer (29-32). While numerous studies have investigated the expression of HOTAIR and miR-646 in EC tissue samples, information regarding their circulating levels in serum is still insufficient (33,34).

ncRNA species, such as miRs, lncRNAs and circular RNAs (circRNAs), serve crucial roles in the positive and negative regulation of the expression of genes. Over the past two decades, numerous studies highlight the involvement of ncRNAs in various aspects of cell biology, particularly in cancer development, progression and drug resistance (43-48).

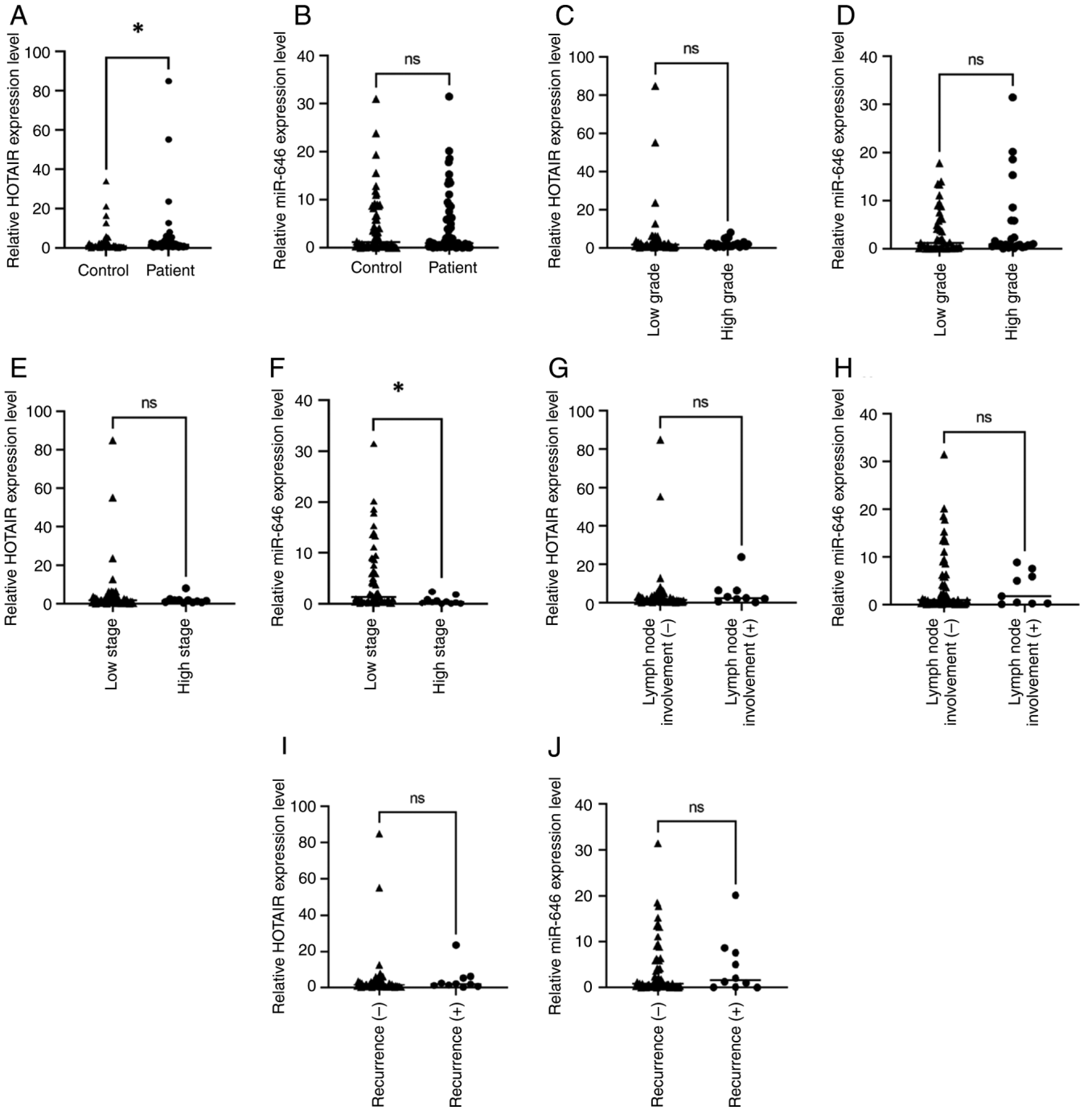

Figure 1. Differential expression levels of HOTAIR and miR-646 according to tumour grade, stage, lymph node involvement and recurrence status in patients with endometrial cancer. (A) HOTAIR and (B) miR-646 expression levels in control vs. patient groups. (C) HOTAIR and (D) miR-646 expression levels according to tumour grade. Endometrial cancer was classified as low grade (grades 1-2 endometrioid) and high grade (grade 3 endometrioid and non-endometrioid histology types). (E) HOTAIR and (F) miR-646 expression levels according to tumour stage. Stages IA and B were considered as early stages, while stages II-IV were considered high stages. (G) HOTAIR and (H) miR-646 expression levels according to lymph node involvement (negative vs. positive). (I) HOTAIR and (J) miR-646 expression levels according to recurrence status (recurrence-negative vs. recurrence-positive). *P<0.05. HOTAIR, Hox antisense intergenic RNA; miR, microRNA; ns, not significant.

These molecules participate in virtually all cellular processes and have fundamental roles at every stage of tumorigenesis. Altered expression levels of mRNAs, lncRNAs, circRNAs and miRs are reported in numerous types of cancer, such as triple-negative and oestrogen receptor-positive breast cancer, serving as potential biomarkers for diagnosis and therapeutic targets (43,47-50). Furthermore, ncRNAs have been studied as promising biomarkers for early detection and prognostic evaluation in various malignancies, including lung (26), gastric (31), breast (43), prostate (51), liver (52), colorectal (53), bladder (54), pancreatic (55) and haematological disorders (56). This evidence suggests that circulating lncRNAs in peripheral blood may potentially serve as non-invasive biomarkers for cancer diagnosis and monitoring.

The expression of miR is tissue-specific and serves a key role in maintaining cellular homeostasis. Their stability in

Table II. Spearman correlation between HOTAIR and miR-646.

Parameter		r-value	P-value
HOTAIR	miR-646	0.105	0.212

HOTAIR, Hox antisense intergenic RNA; miR, microRNA; r, Spearman's correlation coefficient.

Table III. Spearman correlation of HOTAIR and miR-646 with CA125.

	Correlation with CA125	
Parameter	r-value	P-value
HOTAIR	0.091	0.288
miR-646	0.053	0.385

HOTAIR, Hox antisense intergenic RNA; miR, microRNA; CA125, cancer antigen 125; r, Spearman's correlation coefficient.

circulation and cancer-specific expression patterns have led to their investigation as promising non-invasive biomarkers in various malignancies including breast, lung, colorectal and prostate cancer (57-61). Although the majority of studies focus on other types of cancer, such as ovarian, breast and colorectal cancer (59-61), evidence suggests that specific miRs and lncRNAs exhibit altered expression profiles in EC tissues compared with normal endometrium, indicating potential roles in diagnosis and prognosis (62).

HOTAIR is an important prognostic biomarker that is overexpressed in various types of cancer including breast, ovarian, colorectal and lung cancer (16-20). Its increased expression level is linked to higher levels of suppressor of zeste 12 (SUZ12), a gene involved in epigenetic regulation (63). This suggests that HOTAIR may serve a role in modifying chromatin structure in tumours (64,65). HOTAIR may also mediate genome-wide epigenetic changes by recruiting chromatin-modifying complexes such as the polycomb repressive complex 2 (containing enhancer of zeste homolog 2, SUZ12 and embryonic ectoderm development), which catalyses histone 3 lysine 27 trimethylation, and the lysine specific demethylase 1/corepressor for element-1-silencing transcription factor/RE1-silencing transcription factor complex, which demethylates the active histone hallmark histone H3 lysine 4 trimethylation (63,66). This mechanism may explain its overexpression in breast, colorectal, hepatocellular, gastrointestinal and pancreatic tumours (66). Located within the HOXC gene cluster, HOTAIR enhances tumour invasiveness and metastasis (63). Furthermore, Bhan *et al* (67) reports that the expression of HOTAIR is transcriptionally regulated by oestradiol. HOTAIR is similarly upregulated in EC in tumour tissues compared with normal endometrium. The expression of HOTAIR is associated with higher tumour grades, lymph node metastasis and poor clinical prognosis (21,22,68). Functional studies demonstrate that downregulation of HOTAIR can inhibit cell proliferation and invasion *in vitro* and *in vivo* (68,69). Furthermore, HOTAIR contributes to chemoresistance by promoting cisplatin resistance by regulating autophagy pathways in EC cells (70).

In the present study, serum HOTAIR expression levels were significantly increased in patients with EC compared with the control group. Although this difference was statistically significant, its diagnostic accuracy (sensitivity and specificity) was modest, limiting its immediate clinical applicability. In contrast to previous studies on tumour tissue (17,22,33,68-70) the results of the present study demonstrated no correlation between serum HOTAIR expression levels and disease stage, tumour grade or lymph node metastasis. Additionally, survival analysis revealed no significant association with patient outcomes. These discrepancies may be attributed to differences in the patient populations, or they may indicate that tissue-level associations may not be fully captured in serum-based assessments.

miR-646 is associated with numerous types of cancer including non-small cell lung, clear cell renal, retinoblastoma, laryngeal squamous cell, colorectal, gastric and breast cancer and is generally downregulated in malignancies, where it contributes to tumour growth, metastasis and invasion (25-31). By contrast, its upregulation suppresses these processes. Previous studies by Liu *et al* (32) and Zhou *et al* (33) demonstrate that miR-646 inhibits the expression of NPM1 and exerts tumour-suppressive effects in EC cell lines including HEC-1A and Ishikawa. The study by Zhou *et al* (33) demonstrates that miR-646 expression levels are reduced in both EC tissues and cancer cell lines compared with normal endometrial tissues and cells, respectively. Furthermore, HOTAIR reduces miR-646 expression levels *in vitro*, and a positive association between HOTAIR and NPM1, alongside a negative association between NPM1 and miR-646, is observed in EC tissues (32,33).

The results of the present study found no statistically significant difference in serum miR-646 expression levels between patients with EC and healthy controls. Additionally, miR-646 expression levels were not demonstrated to be associated with tumour grade, recurrence, lymph node metastasis or overall survival. However, patients with advanced-stage EC exhibited significantly lower miR-646 levels compared with those with early-stage EC. This suggested a possible association between miR-646 downregulation and disease progression, which may be potentially mediated through the NPM1 pathway. However, the expression of NPM1 was not assessed in the present investigation.

The associations between serum levels of HOTAIR and miR-646 with CA-125 were further investigated; however, no significant associations were revealed. ROC curve analysis of HOTAIR revealed an area under the curve of 0.617, with an optimal threshold value of 1.08, sensitivity of 63.3% and specificity of 61.2%. This suggested a modest diagnostic accuracy. Although these values indicated a modest diagnostic accuracy, they fall below the commonly accepted criteria for clinical applicability (AUC >0.80; sensitivity and specificity >80%) (71), which suggested that serum HOTAIR alone was insufficient for diagnostic use; however, it may serve as a potential adjunct biomarker. Future research focusing on exosomal HOTAIR expression levels may enhance diagnostic performance.

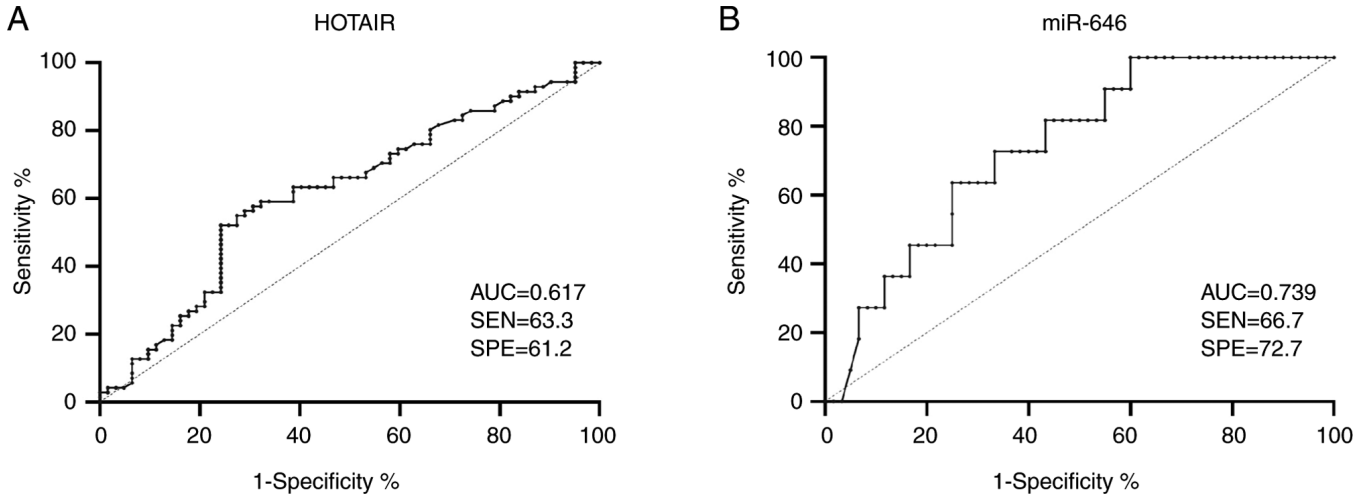

Figure 2. Receiver operating characteristic curve analyses for serum HOTAIR and miR-646. (A) To distinguish patients with endometrial cancer from healthy controls, the cut-off value for HOTAIR was 1.08. (B) To distinguish advanced from early-stage endometrial cancer, the cut-off value for miR-646 was 0.655. HOTAIR, Hox antisense intergenic RNA; miR, microRNA; AUC, area under the curve; SEN, sensitivity; SPE, specificity.

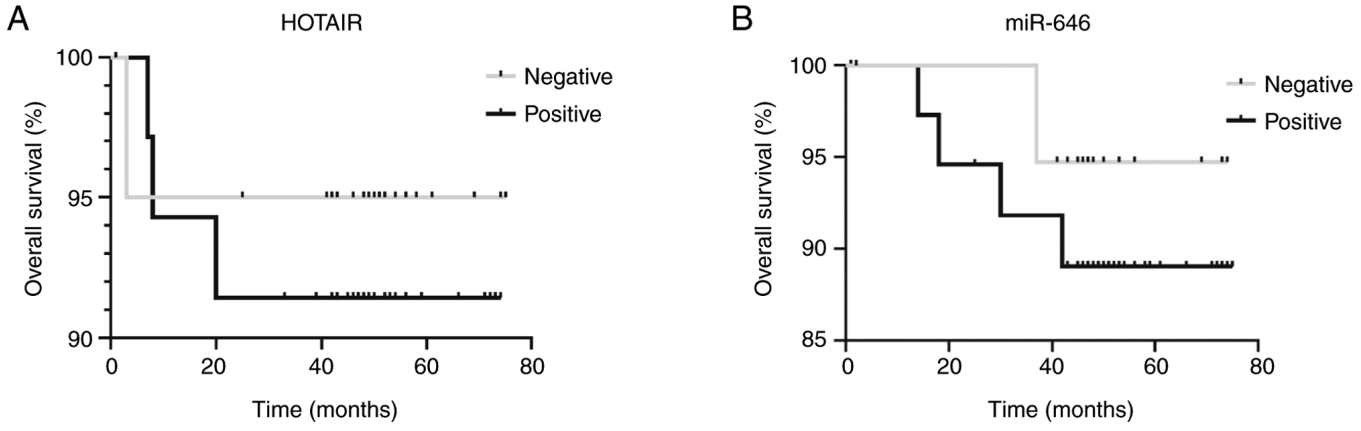

Figure 3. Overall survival analysis according to serum HOTAIR and miR-646 expression levels. Patients with endometrial cancer were divided into high- or low-expression groups based on median relative expression levels. (A) Patients were classed as 'negative' or 'positive' if the median relative HOTAIR expression levels were <1.08 or ≥1.08, respectively. (B) Patients were classed as 'negative' or 'positive' if the median relative miR-646 expression levels were <0.655 or ≥0.655, respectively. HOTAIR, Hox antisense intergenic RNA; miR, microRNA.

Regarding miR-646, ROC analysis differentiating early from advanced stages of EC demonstrated an optimal cut-off value of 0.655, with sensitivity and specificity values of 66.7 and 72.7%, respectively. Although these metrics were below the levels required for a clinically useful diagnostic test, they suggested that miR-646 may have a potential utility in disease staging, particularly if exosomal miR-646 expression levels are investigated in future studies. Although there was not a significant association between HOTAIR or miR-646 levels and tumour grade, lymph node metastasis or recurrence status, these findings should be interpreted cautiously. It is possible that serum-based assessments do not accurately reflect the tumour microenvironment, or that the small sample size of the present study limited the ability of the present study to detect these associations. The novelty of the present study was the evaluation of serum-based expression levels of HOTAIR and miR-646 as potential non-invasive biomarkers in EC, which, to the best of our knowledge, has not been previously reported in clinical patient samples.

The present study had several limitations. Firstly, in the present study, the expression of HOTAIR or miR-646 in exosomes was not assessed, which may provide a more accurate reflection of tumour-derived ncRNAs. Secondly, the small number of patients with lymph node metastasis and recurrence limited the possibility of conducting subgroup analyses. Thirdly, the absence of associations between miR-646 and certain clinicopathological features may be due to the characteristics of the cohort used in the present study or the lack of exosomal data. Finally, the use of GAPDH and U6 as endogenous controls for normalization may represent another limitation of the present study. Although these reference genes are used in serum-based analyses of lncRNA and miR to ensure comparability with previous studies, their stability in circulation is not absolute and may introduce technical variability. Future studies should incorporate spike-in controls, such as synthetic cel-miR-39 or Universal spike-in oligonucleotides, for both HOTAIR and miR-646 to monitor RNA extraction and reverse transcription

efficiency (72), and use stable endogenous RNAs (such as U6 for miR-646 (73) and GAPDH or 18S ribosomal RNA for HOTAIR (74) to improve normalisation and enhance the reproducibility of results. In conclusion, the results of the present study reinforced the relevance of HOTAIR and miR-646 in EC. Serum HOTAIR may contribute to diagnosis, while serum miR-646 may reflect disease stage. However, both biomarkers require validation in larger, more diverse patient cohorts and studies incorporating exosomal analysis in order to clarify their clinical applicability in the diagnosis, monitoring and prognosis of EC. In addition to their potential use as biomarkers, the findings of the present study suggested that both HOTAIR and miR-646 may serve as targets for new treatment strategies in EC. The increased expression of HOTAIR could possibly be reduced by drugs that block its function, and the decreased expression of miR-646 in advanced stages may possibly be restored using miR-646 mimics. The results of the present study may be useful not only for diagnosis and prognosis but also for the development of novel therapies.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

RH, HK, YM, HS, ST, CK and AFA conceived and designed the experiments. RH, HK, YM, HS, ST, CK and AFA confirm the authenticity of all the raw data. RH, HK, YM, HS, ST, CK and AFA performed the experiments, including literature database searching where applicable, conducted data analysis, prepared figures and tables, and contributed to the drafting of the manuscript and its critical revision for important intellectual content. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Istanbul Faculty of Medicine, Istanbul University (approval date, 01.07.2021; approval no. 272576; Istanbul, Turkey). Written informed consent was obtained from all participants, and the study was conducted in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Use of artificial intelligence tools

During the preparation of this work, artificial intelligence tools were used to improve the readability and language of the manuscript or to generate images, and subsequently, the authors revised and edited the content produced by the artificial intelligence tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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