Combination of circulating tumor cells, lncRNAs and DNA methylation for the diagnosis of endometrial carcinoma

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Abstract. Endometrial carcinoma (EC) is one of the most common gynecological malignant neoplasms, the prognosis of which is strongly related to the time of diagnosis, with an earlier diagnosis leading to a better prognosis. Therefore, effective diagnostic indicators and methods are needed to ensure early detection. The present study explored the following in EC: Circulating tumor cells (CTCs); the long noncoding RNAs (lncRNAs) RP4-616B8.5, RP11-389G6.3 and carboxy-terminal domain (CTD)-2377D24.6; and the methylation of cysteine dioxygenase type 1 (*CDO1*) and CUGBP Elav-like family member 4 (*CELF4*). In total, 85 patients, including 71 with EC, and 14 without EC (NO-EC) but with uterine fibroids or polyps, were included in the present study. In total, 46 patients with EC and 8 NO-EC patients underwent CTC detection. In the evaluation of the EC vs. NO-EC groups, the results showed that the CTC-positive rate of the EC group was 80.43% and that the area under the curve (AUC) value of CTCs was 0.8872 (P=0.0098). A total of 35 patients with EC and 14 NO-EC patients underwent detection of the RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 lncRNAs. When the levels of the three lncRNAs RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 were compared between the EC and NO-EC groups, they were higher in the EC group; the P-values were 0.0002, 0.0001 and <0.0001, respectively, and the AUC values were 0.8184, 0.8347 and 0.8265, respectively. In addition, a total of 35 patients with EC and 8 NO-EC patients underwent *CDO1* and *CELF4* DNA methylation analysis. The positive rates of the methylated genes *CDO1* and *CELF4* were 20% (7/35) and 5.71% (2/35), and the P-values of the comparisons between the EC and NO-EC groups were 0.1748 and 0.5004, respectively; the AUC values were 0.6000 and 0.5286. Furthermore, the combination of CTCs, and lncRNAs RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 exhibited high performance in the detection of EC (AUC=0.9375).

Introduction

Endometrial carcinoma (EC) is one of the most common gynecological malignancies in developed countries (1). Notably, the incidence of EC has been increasing in a number of countries, including the United States, as well as in Europe and East Asia, which may be due to a greater exposure to environmental risk factors, such as obesity, increasing age (≥55 years) and a shift in female reproductive patterns (2-4). Moreover, the incidence of EC is estimated to increase by 55% from 2010 to 2030 (5).

Current clinical data have indicated that the prognosis of EC is closely related to the time of diagnosis, with an earlier diagnosis associated with a better prognosis [International Federation of Gynecology and Obstetrics (FIGO) stage I-II]; for example, the 5-year survival rate has been reported to decrease from 85% for stage I disease to 25% for stage IV disease (6-8). Currently, EC is diagnosed by a combination of transvaginal ultrasound (TVUS) and endometrial biopsy; however, there is marked heterogeneity in the accuracy of TVUS for detecting malignancies, as its sensitivity ranges from 0.5 to 0.8 for gynecologic oncological diseases (9). On the other hand, endometrial biopsy is invasive and uncomfortable for the patient, and pathological assessment sometimes cannot be carried out due to the failure of the sampling owing to the pain of the sampling process or problems of cervical stenosis (10). Furthermore, the role of test results in guiding personalized treatment plans requires more research support. There are still unanswered questions regarding EC, including a number in the domains of treatment toxicity, diagnostic procedures and adjuvant therapy (11,12). Therefore, reliable

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detection of EC is necessary to ensure adequate treatment and reduce EC-associated mortality.

With recent advancements in technology, researchers have focused on developing robust and sensitive detection methods, such as circulating tumor cell (CTC) detection, as well as methods involving genomics, epigenomics and transcriptomics (13,14). CTCs disseminate into the bloodstream from either the primary tumor site or metastatic sites (15). Consequently, the number of CTCs is higher in patients with various types of cancer, such as lung cancer and breast cancer, than in healthy volunteers (16,17). In a study of the detection of EC CTCs, aminopeptidase N (CD13) was identified as an alternative prognostic marker for both cervical and endometrial cancer, as its expression was detected in patients with EC before surgery and after recurrence (18).

Long noncoding RNAs (lncRNAs) have an important role in the epigenetic regulatory network, and can regulate gene expression and post-transcriptional processes by influencing the structures of protomers, chromatin and transcription factors (19). A number of studies have reported that some lncRNAs affect various hallmarks of human cancer, such as replicative immortality, antagonism of cell death and evasion of immunosurveillance (20,21); therefore, lncRNAs, such as RP4-616B8.5, RP11-389G6.3, carboxy-terminal domain (CTD)-2377D24.6, AC138904.1 and AC099329.2, are used as biomarkers in numerous cancer diagnoses (22,23). Ding *et al* (24) reported that the combination of lncRNAs RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 had good performance (P<0.0001) in EC diagnosis. Xin *et al* (25) reported that low RP11-395G23.3 expression was significantly associated with advanced histological grade and lymphovascular space invasion in patients with EC, and that RP11-395G23.3 may be a target for the diagnosis and treatment of EC.

Cytosine methylation of DNA within CpG dinucleotides is the most well-researched epigenetic alteration in humans (26). Hypermethylation of the CpG islands of gene promoters can silence genes, and this is the basis of the clinical use of a number of biomarkers (27,28). DNA methylation is a highly stable molecular feature that can be detected in tumor tissues and cells (29,30). During malignant transformation, EC cells acquire two main types of aberrant DNA methylation patterns: Local DNA hypermethylation and global DNA hypomethylation (31). Qi *et al* (32) reported that hypermethylated cysteine dioxygenase type 1 (*CDO1*) and CUGBP Elav-like family member 4 (*CELF4*) could serve as triage strategy biomarkers in the non-invasive examination of endometrial malignant lesions, and the sensitivity and specificity of *CDO1*/*CELF4* dual-gene methylation assay for endometrial atypical hyperplasia and endometrial cancer reached 84.9 and 86.6%, respectively. *CDO1* and zinc finger protein 454 hypermethylation has also been verified in histological samples from patients with EC and atypical hyperplasia (AH) compared with those from patients with benign and normal endometria (P<0.001) (33).

To increase the efficacy of EC screening, a combination of major biomarkers, namely, CTCs, lncRNAs (RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6) and DNA methylation (*CDO1* and *CELF4*), was evaluated in the present study to construct a better diagnostic model for EC.

Materials and methods

Specimens. A total of 85 patients, including 71 with EC, and 14 without EC (NO-EC) but with uterine fibroids or polyps, were enrolled from The First Affiliated Hospital of Soochow University (Suzhou, China) between March 2023 and March 2024. All enrolled patients were female; aged 32-84 years (mean age, 57.75 years); and had provided written informed consent before participation in the present study, with permission given for sample collection and analysis. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Soochow University (approval no. 2021.351). The diagnostic criteria for EC were based on the 2014 World Health Organization Classification of Tumours of the Female Reproductive Organs. The samples were collected before any anticancer drug treatment. The specific clinical information of the subjects is shown in Table I and the groups are shown in Fig. 1.

CTC enrichment and detection. A total of 46 patients with EC and 8 NO-EC patients underwent CTC detection. Peripheral blood (PB) samples (4 ml/patient) were collected before surgery or treatment, stored in EDTA tubes (Becton, Dickinson and Company) and CTCs were detected within 6 h using the CytoBot® 2000 system (Holosensor Medical Technology Ltd.). Before CTC detection, PB mononuclear cells (PBMCs) were isolated from the PB. Briefly, 4 ml density gradient separation solution (Shenzhen DAKEWE Bio-engineering Co., Ltd.) and a diluted blood sample (4 ml PB with an equal volume of phosphate buffer, pH 7.0; Biological Industries) were added sequentially to a sterile 15-ml centrifuge tube and centrifuged at 700 x g for 20 min at room temperature. The PBMCs were then carefully pipetted into a new 15-ml centrifuge tube, washed twice with 5-10 ml PBS (pH 7.2) and centrifuged at 500 x g for 5 min at 25˚C.

CTCs were detected using the CytoBot 2000 system, a novel CTC platform based on advanced technology, including microfluidics and immunoenrichment. Briefly, CTC capture chips were manufactured using a metal mesh with pores measuring 15 μm in diameter, and gold-covered polymers and the purified anti-human CD326 (Ep-CAM) capture antibody (cat. no. 324202; BioLegend, Inc.) were seeded onto the surface to form a capture chip with unique functionality. In the present study, the PBMCs were resuspended in PBS (pH 7.2) to a volume of 300 μl and were loaded onto the capture chip. CTCs were captured and stained by the CytoBot® 2000 system using the preset procedures and pre-prepared reagents from the CTCs detection kit (Holosensor, Inc.).

The immunofluorescence staining was carried out using the CytoBot 2000 system and the CTCs detection kit (Holosensor, Inc.), and the indicators used were Alexa Fluor® 488 anti-pancytokeratin (CK), PE anti-human CD45 antibodies and DAPI. The cell types were determined under a fluorescence microscope [RX50M; Sunny Optical Technology (Group) Company Limited]. The evaluation criteria of CTCs was $CK^+CD45^-DAPI^+$, and the threshold for CTC positivity was a $CK^+CD45^-DAPI^+$ CTC number of ≥2.

Tissue sample collection, RNA isolation and reverse transcription-quantitative PCR (RT-qPCR) analysis. In total, 35 patients with EC and 14 NO-EC patients underwent

Table I. Patient information.

Characteristic	Patients with EC[a] (n=71)			NO-EC[a] patients (n=14)		EC vs. NO-EC
	Endometrioid adenocarcinoma (n=55)	Serous adenocarcinoma (n=9)	Other EC (n=7)	Uterine fibroids (n=10)	Polyps (n=4)	P-value
Mean ± SD age, years	57.80±10.20	59.11±8.824	57.8±9.822	53.70±8.001	64.00±10.00	0.6448
Hypertension						
Yes	33/55 (60.00%)	2/9 (22.22%)	3/7 (42.86%)	2/10 (20.00%)	1/4 (25.00%)	0.0281
No	22/55 (40.00%)	7/9 (77.78%)	4/7 (57.14%)	8/10 (80.00%)	3/4 (75.00%)	
Diabetes						
Yes	13/55 (23.64%)	2/9 (22.22%)	1/7 (14.29%)	0/10 (0.00%)	0/4 (0.00%)	0.0494
No	42/55 (76.36%)	7/9 (77.78%)	6/7 (85.71%)	10/10 (100.00%)	4/4 (100.00%)	
Fatty liver						
Yes	19/55 (34.55%)	3/9 (33.33%)	0/7 (0.00%)	1/10 (10.00%)	2/4 (50.00%)	0.4791
No	36/55 (65.45%)	6/9 (66.67%)	7/7 (100.00%)	9/10 (90.00%)	2/4 (50.00%)	
LDL[a]						
High (>3.4 mmol/l)	19/55 (34.55%)	5/9 (55.56%)	5/7 (71.43%)	1/10 (10.00%)	2/4 (50.00%)	0.1746
Normal (≤3.4 mmol/l)	36/55 (65.45%)	4/9 (44.44%)	2/7 (28.57%)	9/10 (90.00%)	2/4 (50.00%)	
HDL[a]						
Low (<1.0 mmol/l)	16/55 (29.09%)	1/9 (11.11%)	2/7 (28.57%)	1/10 (10.00%)	0/4 (0.00%)	0.1165
Normal (≥1.0 mmol/l)	39/55 (70.91%)	8/9 (88.89%)	5/7 (71.43%)	9/10 (90.00%)	4/4 (100.00%)	
TAG[a]						
High (>1.7 mmol/l)	20/55 (36.36%)	5/9 (55.56%)	5/7 (71.43%)	3/10 (30.00%)	1/4 (25.00%)	0.8599
Normal (≤1.7 mmol/l)	35/55 (63.64%)	4/9 (44.44%)	2/7 (28.57%)	7/10 (70.00%)	3/4 (75.00%)	
Cholesterol						
High (>5.2 mmol/l)	19/55 (34.55%)	5/9 (55.56%)	3/7 (42.86%)	2/10 (20.00%)	1/4 (25.00%)	0.2399
Normal (≤5.2 mmol/l)	36/55 (65.45%)	4/9 (44.44%)	4/7 (57.14%)	8/10 (80.00%)	3/4 (75.00%)	
HE4[a]						
High (>70 pmol/l, before menopause; >140 pmol/l, post-menopause)	18/55 (32.73%)	1/9 (11.11%)	2/7 (28.57%)	0/10 (00.00%)	0/4 (0.00%)	0.0188
Normal (≤70 pmol/l, before menopause; ≤140 pmol/l, post-menopause)	37/55 (67.27%)	8/9 (88.89%)	5/7 (71.43%)	10/10 (100.00%)	4/4 (100.00%)	
Glucose						
Normal (3.9-6.1 mmol/l)	16/55 (29.09%)	1/9 (11.11%)	1/7 (14.29%)	1/10 (10.00%)	0/4 (0.00%)	0.1165
High (>6.1 mmol/l)	39/55 (70.91%)	8/9 (88.89%)	6/7 (85.71%)	9/10 (90.00%)	4/4 (100.00%)	

Table I. Continued.

Characteristic	Patients with EC[a] (n=71)			NO-EC[a] patients (n=14)		EC vs. NO-EC
	Endometrioid adenocarcinoma (n=55)	Serous adenocarcinoma (n=9)	Other EC (n=7)	Uterine fibroids (n=10)	Polyps (n=4)	P-value
Number of pregnancies						
0	3/55 (5.45%)	0/9 (0.00%)	0/7 (0.00%)	0/10 (0.00%)	0/4 (0.00%)	0.4257
1	12/55 (21.82%)	2/9 (22.22%)	0/7 (0.00%)	3/10 (30.00%)	1/4 (25.00%)	
2	14/55 (25.45%)	3/9 (33.33%)	4/7 (57.14%)	4/10 (40.00%)	2/4 (50.00%)	
3	12/55 (21.82%)	3/9 (33.33%)	1/7 (14.29%)	1/10 (10.00%)	0/4 (0.00%)	
≥4	14/55 (25.45%)	1/9 (11.11%)	2/7 (28.57%)	2/10 (20.00%)	1/4 (25.00%)	
Stage						
Stage I	48/55 (87.27%)	5/9 (55.56%)	3/7 (42.86%)			
Stage II	4/55 (7.27%)	1/9 (11.11%)	3/7 (42.86%)			
Stage III	3/55 (5.45%)	1/9 (11.11%)	1/7 (14.29%)			
Stage IV	0/55 (0.00%)	2/9 (22.22%)	0/7 (0.00%)			
Muscular layer infiltration depth						
<1/2	44/55 (80.00%)	6/9 (66.67%)	4/7 (57.14%)			
≥1/2	11/55 (20.00%)	3/9 (33.33%)	3/7 (42.86%)			
Tumor size, cm						
<2	16/55 (29.09%)	3/9 (33.33%)	2/7 (28.57%)			
≥2	39/55 (70.91%)	6/9 (66.67%)	5/7 (71.43%)			
HPV[a]						
Positive	6/31 (19.35%)	4/8 (50.00%)	2/6 (33.33%)		0/3 (0.00%)	0.3695
Negative	25/31 (80.65%)	4/8 (50.00%)	4/6 (66.67%)		3/3 (100.00%)	
CEA[a]						
High (>5 ng/ml, no smoking; >10 ng/ml, smoking)	1/39 (2.57%)	0/7 (0.00%)	0/3 (0.00%)	0/10 (0.00%)	1/3 (33.33%)	0.3131
Normal (0-5 ng/ml, no smoking; 0-10 ng/ml, smoking)	38/39 (97.43%)	7/7 (100.00%)	3/3 (100.00%)	10/10 (100.00%)	2/3 (66.67%)	
CA19-9[a]						
High (>37 U/ml)	9/51 (17.65%)	1/8 (12.50%)	0/6 (0.00%)	1/10 (10.00%)	0/3 (0.00%)	0.4734
Normal (0-37 U/ml)	42/51 (82.35%)	7/8 (87.50%)	6/6 (100.00%)	9/10 (90.00%)	3/3 (100.00%)	
CA125[a]						
High (>35 U/ml)	9/53 (16.98%)	2/9 (22.22%)	0/6 (0.00%)	2/10 (20.00%)	0/2 (0.00%)	0.9667
Normal (0-35 U/ml)	44/53 (83.02%)	7/9 (77.78%)	6/6 (100.00%)	8/10 (80.00%)	2/2 (100.00%)	

Table I. Continued.

Characteristic	Patients with EC[a] (n=71)			NO-EC[a] patients (n=14)		EC vs. NO-EC
	Endometrioid adenocarcinoma (n=55)	Serous adenocarcinoma (n=9)	Other EC (n=7)	Uterine fibroids (n=10)	Polyps (n=4)	P-value
ER[a]						
Positive	44/50 (88.00%)	7/7 (100.00%)	4/4 (100.00%)			
Negative	6/50 (12.00%)	0/7 (0.00%)	0/4 (0.00%)			
PR[a]						
Positive	42/54 (77.78%)	6/6 (100.00%)	5/5 (100.00%)			
Negative	12/54 (22.22%)	0/6 (0.00%)	0/5 (0.00%)			
Ki67[a]						
Positive	55/55 (100.00%)	7/7 (100.00%)	5/5 (100.00%)			
Negative	0/55 (0.00%)	0/7 (0.00%)	0/5 (0.00%)			
MSH2[a]						
Positive	28/28 (100.00%)	4/4 (100.00%)	3/3 (100.00%)			
Negative	0/28 (0.00%)	0/4 (0.00%)	0/3 (0.00%)			
CTC[a]						
Positive	31/36 (86.11%)	4/8 (50.00%)	2/2 (100.00%)	0/8 (0.00%)		0.0098
Negative	5/36 (13.89%)	4/8 (50.00%)	0/2 (0.00%)	8/8 (100.00%)		
CDO1 DNA methylation[a]						
Positive	7/29 (24.14%)	0/5 (0.00%)	0/1 (0.00%)	0/8 (0.00%)		0.1748
Negative	22/29 (75.86%)	5/5 (100.00%)	1/1 (100.00%)	8/8 (100.00%)		
CELF4 DNA methylation[a]						
Positive	2/29 (6.90%)	0/5 (0.00%)	0/1 (0.00%)	0/8 (0.00%)		0.5004
Negative	27/29 (93.10%)	5/5 (100.00%)	1/1 (100.00%)	8/8 (100.00%)		

[a]Detected in some patients. *CDO1*, cysteine dioxygenase type 1; CA, cancer antigen; CEA, carcinoembryonic antigen; *CELF4*, CUGBP Elav-like family member 4; CTC, circulating tumor cell; EC, endometrial carcinoma; ER, estrogen receptor; HDL, high-density lipoprotein; HE4, human epididymis protein 4; LDL, low-density lipoprotein; NO-EC, without EC; PR, progesterone receptor.

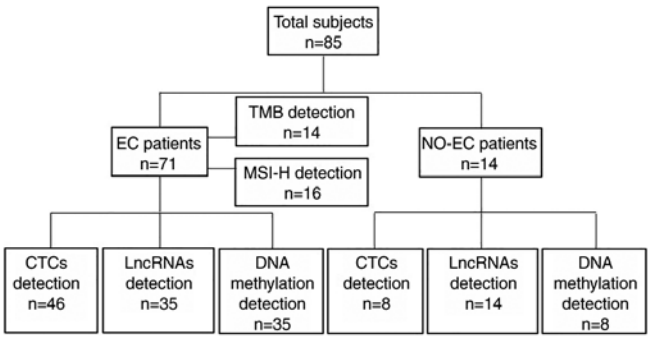

Figure 1. Patient groups. A total of 85 patients, including 71 patients with EC and 14 NO-EC patients with uterine fibroids or polyps, were included. A total of 46 patients with EC and 8 NO-EC patients underwent CTC detection. A total of 35 patients with EC and 14 NO-EC patients underwent RP4-616B8.5, RP11-389G6.3 and carboxy-terminal domain-2377D24.6 lncRNA detection. A total of 35 patients with EC and 8 NO-EC patients underwent cysteine dioxygenase type 1 and CUGBP Elav-like family member 4 DNA methylation analysis. Of the patients with EC, 14 underwent TMB analysis and 16 underwent MSI-H analysis. CTC, circulating tumor cell; EC, endometrial carcinoma; lncRNA, long noncoding RNA; MSI-H, microsatellite instability-high; NO-EC, without EC; TMB, tumor mutational burden.

RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 lncRNA detection. Tumor tissues, paracancerous tissues (at a 1-cm distance from tumor tissues), uterine fibroid and polyp tissues were obtained during surgery before treatment. Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and RNA was subsequently reverse transcribed into cDNA with a PrimeScript RT reagent kit (Takara Biotechnology, Ltd.) according to the manufacturer's protocol. The expression levels of RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 lncRNAs were measured by qPCR using the Hiff qPCR SYBR Green Master Mix (Shanghai Yeasen Biotechnology Co., Ltd.) and the QuantStudio 6 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR conditions were as follows: Pre-denaturation at 95˚C for 10 min; followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 sec and extension at 70˚C for 30 sec; and a hold at 95˚C for 15 sec, 60˚C for 1 min. The positive standard was a Cq value of ≤40. The expression levels were normalized to the levels of *GAPDH* mRNA and were calculated using the ΔCq method (34). The primers used for analysis are listed in Table II.

CDO1 and CELF4 DNA methylation analysis. In total, 35 patients with EC and 8 NO-EC patients were included in this analysis. For clinical testing, cervical epithelial cells and endocervix cells were collected using a cervical brush or cervical epidermal cell sampler. In this experiment, the cervical epidermal cell samples were scraped from subjects with endometrial cell collectors (SAP-I) and were placed in sample preservation solution (cat. no. AM7020; Thermo Fisher Scientific, Inc.). Genomic DNA was extracted using the TIANamp Genomic DNA Kit (cat. no. DP304; Tiangen Biotech Co., Ltd.) and 20 μl DNA eluent was obtained.

A custom-developed bisulfite conversion kit (methylation detection sample pretreatment kit; Holosensor Medical Technology Ltd.) was used to convert the extracted DNA into bisulfite and obtain the transformed bis-DNA. Finally, *CDO1* and *CELF4* amplification was performed on an ABI 7500 device (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction mixture consisted of the PCR solution and primer probes, and the transformed bis-DNA samples were added to the mixture. The reaction conditions were as follows: Pre-denaturation at 96˚C for 5 min, followed by 45 cycles of denaturation at 94˚C for 15 sec and annealing at 60˚C for 35 sec, and a hold at 25˚C for 10 min. The positive standard was a Cq value of ≤38. The primers used for the analysis are listed in Table III.

Table II. Primer sequences used for reverse transcription-quantitative PCR.

Primer	Sequence, 5'-3'
CTD-2377D24.6-F	TTCCGGTGTCCAGATGTTCA
CTD-2377D24.6-R	AAGGTGAGTTGGGGAGGATG
RP4-616B8.5-F	ATGAGTGTGGCAGCCTATGT
RP4-616B8.5-R	AACTCCTGACCTCGTGATCC
RP11-389G6.3-F	GGCCTTGAGAGATAGAGGGG
RP11-389G6.3-R	ATACGTCCTTCCCATCCTGC
GAPDH-F	GCACAGTCAAGGCTGAGAATG
GAPDH-R	ATGGTGGTGAAGACGCCAGTA

F, forward; R, reverse.

Table III. Primer sequences used for DNA methylation detection.

Primer	Sequence, 5'-3'
CDO1 F	ATCAACGTTTATATTTTTAAGTTATCG
CDO1 R	GACTTAGACCCTCTACTAATCCG
CDO1 FP	FAM-CATTCTATTTCGGGCGCGGAGATGCGG-BHQ1
CELF4 F	ATCTCCATGTATATAAAGATGGITACG
CELF4 R	GATATAAGAACTATAACTTAATCCG
CELF4 FP	ROX-ATACCTATAACGGGTTCGGTAGTAGTT-BHQ2

CDO1, cysteine dioxygenase type 1; *CELF4*, CUGBP Elav-like family member 4; F, forward; R, reverse; FP, fluorescent probe; FAM, carboxyfluorescein; BHQ, Black Hole Quencher; ROX, Rhodamine X.

Statistical analysis. Statistical analyses, including receiver operating characteristic (ROC) curve analysis, paired Student's t-test and unpaired Student's t-test, were performed using GraphPad Prism 10.1.2 software (Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Diagnostic value of CTC detection. CTC detection, a classic screening method for tumors, has been applied effectively in

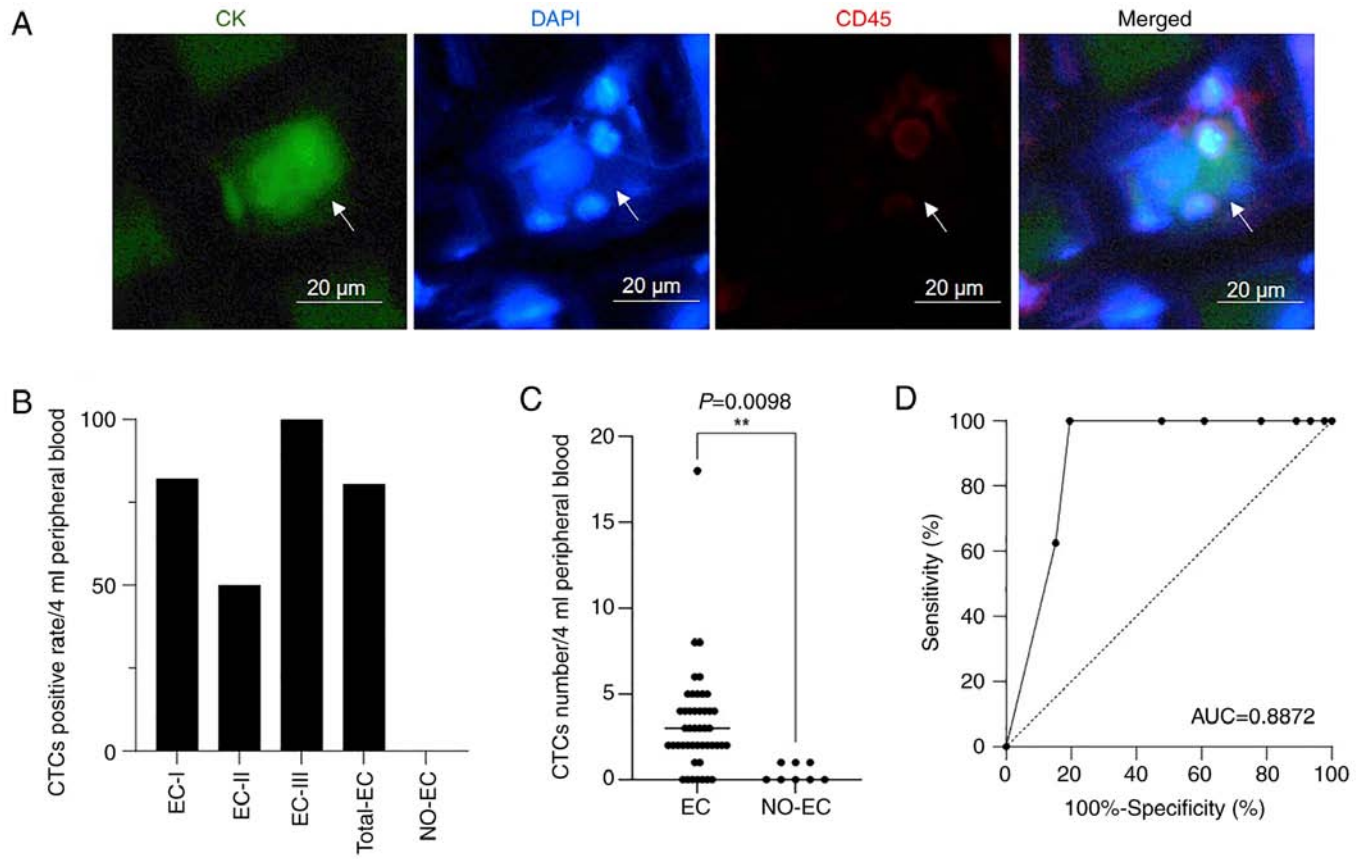

Figure 2. Diagnostic performance of CTCs in EC. (A) CTCs from patients with EC; CK^+ (green), $DAPI^+$ (blue) and $CD45^-$ (red). Scale bars, 20 μm, arrows indicate CTCs. (B) CTC-positive rate per 4 ml peripheral blood in different groups. (C) CTC number per 4 ml peripheral blood from patients with EC (n=46) and NO-EC patients (n=8). **P<0.05. (D) Receiver operating characteristic curve analysis of CTCs between EC (n=46) and NO-EC (n=8) samples. AUC, area under the curve; CK, pan-cytokeratin; CTC, circulating tumor cell; EC, endometrial carcinoma; NO-EC, without EC.

numerous types of cancer (35-37). In the present study, CTC detection was used to evaluate patients with EC and NO-EC patients. The results of CTC enrichment and detection are shown in Fig. 2 and Table I. The classic staining characteristics of the CTCs were $CK^+CD45^-DAPI^+$ (Fig. 2A).

A total of 54 subjects, including 46 patients with EC and 8 NO-EC patients, underwent CTC detection. The total CTC positivity rates for all patients with EC, those with stage I EC, those with stage II EC and those with stage III EC were 80.43% (37/46), 82.05% (32/39), 50% (2/4) and 100% (3/3), respectively (Fig. 2B). In the present study, no patients with stage IV EC underwent CTC detection. Among the 8 NO-EC patients, the CTC positivity rate was 0% (0/8), and the threshold for CTC-positive patients was a $CK^+CD45^-DAPI^+$ CTC number of ≥2. The number of CTCs was significantly increased in patients with EC compared with in NO-EC patients (Fig. 2C). In addition, CTCs performed well in distinguishing between the EC and NO-EC groups, with an area under the curve (AUC) value of 0.8872 (Fig. 2D). These findings indicated that CTCs had a good effect on EC diagnosis.

Diagnostic value of lncRNA detection in EC. Ding *et al* (24) measured the lncRNAs RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 in clinical samples, and reported that they had good diagnostic performance regarding histological subtype (P=0.0001), advanced clinical stage (P=0.011) and clinical grade (P<0.0001) in patients with EC. The present study evaluated the lncRNAs RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 in patients with EC and NO-EC patients by RT-qPCR analysis; however, the results obtained were different from the results of the previous study (24). The expression levels of the RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 lncRNAs were not significantly different between tumor (n=35) and paracancerous (n=35) tissues according to the results of RT-qPCR (P=0.2730, 0.0517 and 0.5180, respectively; Fig. 3A, E and I) and ROC curve analyses (AUC=0.5380, 0.5747 and 0.5192, respectively; Fig. 3C, G and K). However, the performance of RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 in distinguishing the EC group (n=35) from the NO-EC group (n=14) was good (Fig. 3B, F and J), with AUC values of 0.8184, 0.8347 and 0.8265, respectively (Fig. 3D, H and L).

Diagnostic value of CDO1 and CELF4 DNA methylation. DNA methylation detection has been widely used in cancer screening studies (38-40). Huang *et al* (41) reported that a panel comprising any two of the three hypermethylated genes, *BHLHE22*, *CDO1* and *CELF4*, reached a sensitivity of 91.8% and specificity of 95.5%. In view of the good performance that has previously been reported, the present study performed a *CDO1* and *CELF4* DNA methylation analysis. *CDO1* and *CELF4* were detected in 35 EC samples and 8

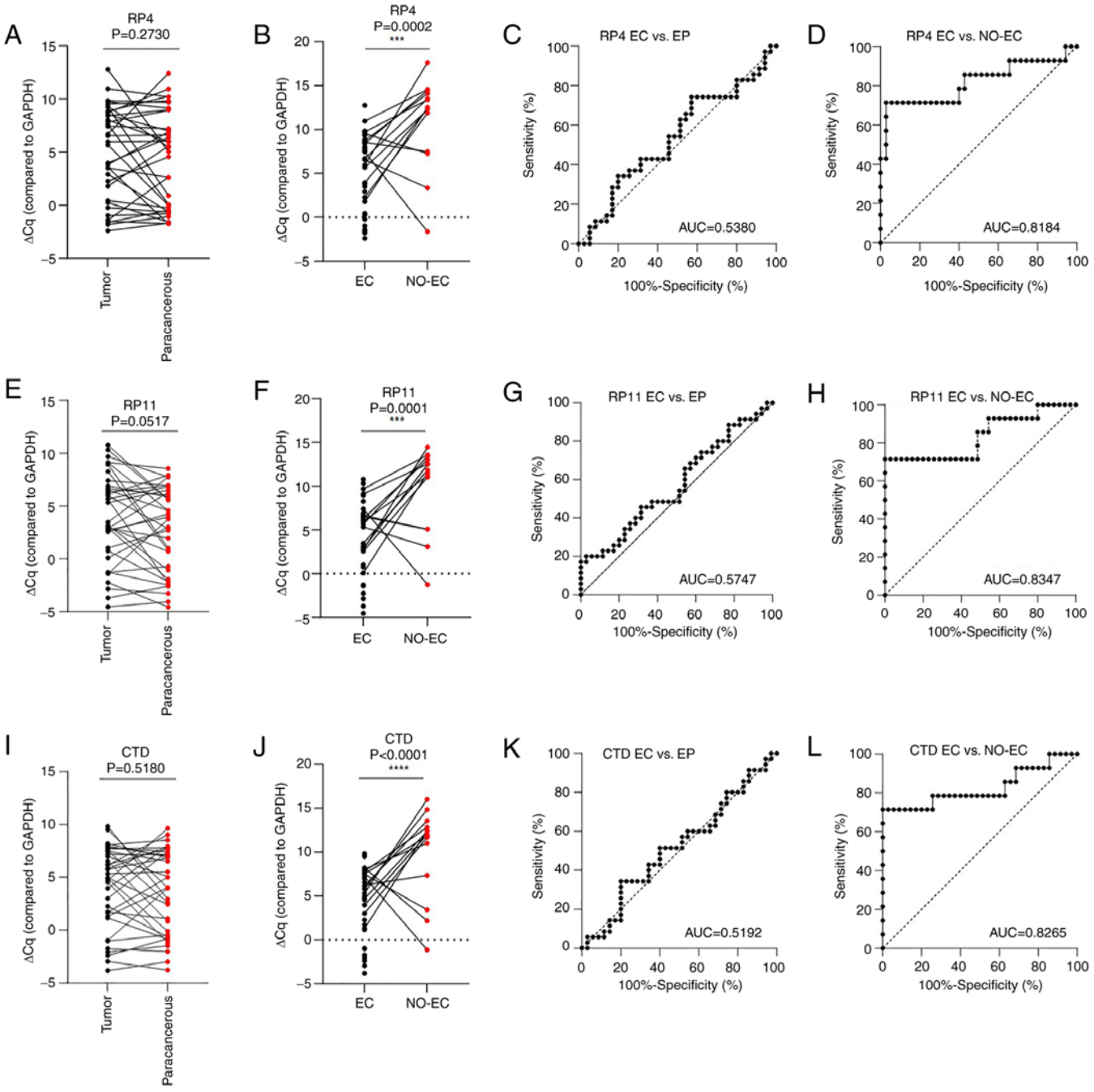

Figure 3. Diagnostic performance of the RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 lncRNAs in EC. ΔCq of RP4-616B8.5 in (A) tumor (n=35) and paracancerous (n=35) tissues, and (B) EC (n=35) and NO-EC (n=14) tissues, as determined by RT-qPCR; *GAPDH* was used as the reference gene. ***P<0.001. ROC analysis of RP4-616B8.5 lncRNA expression between (C) tumor (n=35) and paracancerous (n=35) tissues, and (D) EC (n=35) and NO-EC (n=14) tissues. ΔCq of RP11-389G6.3 lncRNA in (E) tumor (n=35) and paracancerous (n=35) tissues, and (F) EC (n=35) and NO-EC (n=14) tissues, as determined by RT-qPCR; *GAPDH* was used as the reference gene. ***P<0.001. ROC curve analysis of RP11-389G6.3 lncRNA expression between (G) tumor (n=35) and paracancerous (n=35) tissues, and (H) EC (n=35) and NO-EC (n=14) tissues. ΔCq of CTD-2377D24.6 lncRNA in (I) tumor (n=35) and paracancerous (n=35) tissues, and (J) EC (n=35) and NO-EC (n=14) tissues, as determined by RT-qPCR; *GAPDH* was used as the reference gene. ****P<0.0001. ROC curve analysis of CTD-2377D24.6 lncRNA expression between (K) tumor (n=35) and paracancerous (n=35) tissues, and (L) EC (n=35) and NO-EC (n=14) tissues. AUC, area under the curve; CTD, carboxy-terminal domain; EC, endometrial carcinoma; EP, paracancerous tissue; lncRNA, long noncoding RNA; NO-EC, without EC; ROC, receiver operating characteristic.

NO-EC samples (Fig. 1; Table I). The positive rates of *CDO1* and *CELF4* methylation were 20% (7/35) and 5.71% (2/35) in EC, respectively, and these values were lower than those reported in other studies (32,33,40-42). *CDO1* and *CELF4* DNA methylation did not significantly differ between the EC (n=35) and NO-EC (n=8) groups (P=0.1748 and 0.5004, respectively; Table I). In addition, the AUC values were only 0.6000 and 0.5286 for *CDO1* and *CELF4* methylation, respectively (Fig. 4A and B).

To better understand the diagnostic performance of these biomarkers, CTCs and lncRNAs (RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6) were combined, and the AUC value reached 0.9375 (Fig. 4C), thus indicating that CTCs and these lncRNAs had good performance in distinguishing the EC and

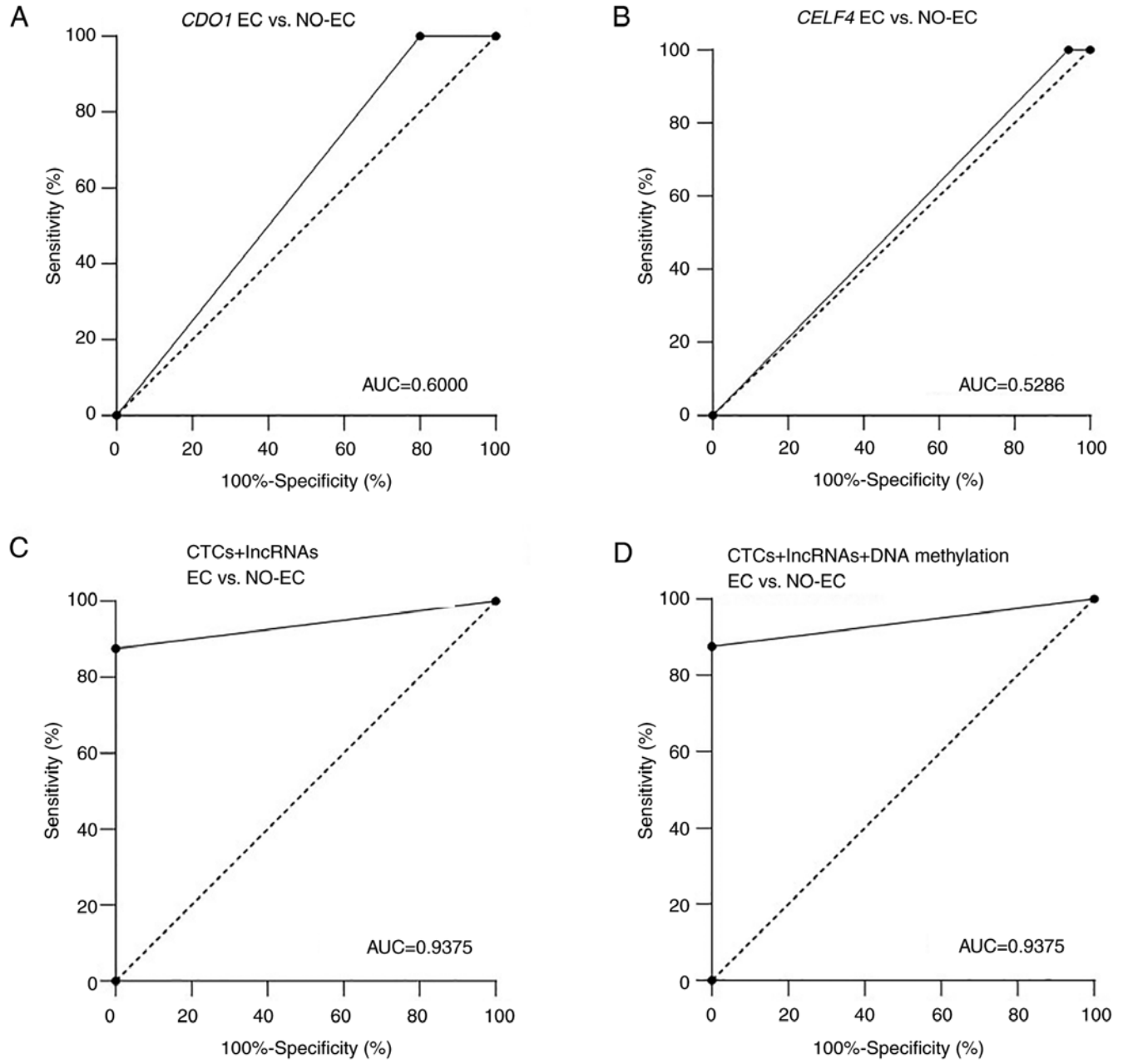

Figure 4. Diagnostic performance of lncRNAs in EC. ROC curve analysis of (A) *CDO1* and (B) *CELF4* DNA methylation in EC (n=35) and NO-EC (n=8) tissue samples. (C) ROC curve analysis of CTCs + lncRNAs (RP4, RP11 and CTD) between EC (n=19) and NO-EC (n=8) samples. (D) ROC curve analysis of CTCs + DNA methylation (*CDO1* and *CELF4*) + lncRNAs (RP4, RP11 and CTD) between EC (n=13) and NO-EC (n=8) samples. AUC, area under the curve; *CDO1*, cysteine dioxygenase type 1; *CELF4*, CUGBP Elav-like family member 4; *CTD*, carboxy-terminal domain; EC, endometrial carcinoma; lncRNA, long noncoding RNA; NO-EC, without EC; ROC, receiver operating characteristic.

NO-EC groups. When all three groups of tumor markers were combined, the AUC value was also 0.9375 (Fig. 4D). These results revealed that the diagnostic performance was not markedly improved after adding methylated genes.

Associations of microsatellite instability-high (MSI-H) status or tumor mutational burden (TMB) with tumor invasiveness and tumor volume. MSI-H and TMB are predictive biomarkers for immune checkpoint inhibitors (43). MSI is an indicator of DNA instability and represents a novel cascade in the carcinogenesis of EC in which MSI mutates hMSH6 (C8), increases gene instability, and leads to the accumulation of mutations in other cancer-related genes (44). The present study investigated the effects of MSI-H status and TMB on EC invasion and tumor volume. TMB was detected in 14 patients with EC, and its association with the depth of muscle infiltration (<1/2 or ≥1/2) or tumor volume (<2 cm or ≥2 cm) was evaluated. The results revealed that high TMB was not significantly correlated with muscle infiltration (P=0.4637; Fig. 5A) or tumor volume (P=0.4637; Fig. 5B). Moreover, MSI-H status was detected in 16 patients with EC, and muscle infiltration (P>0.9999; Fig. 5C) and tumor volume (P=0.1676; Fig. 5D) were not significantly correlated with MSI-H status. These findings indicated that MSI-H status and TMB were not significantly associated with tumor invasion and tumor volume in EC.

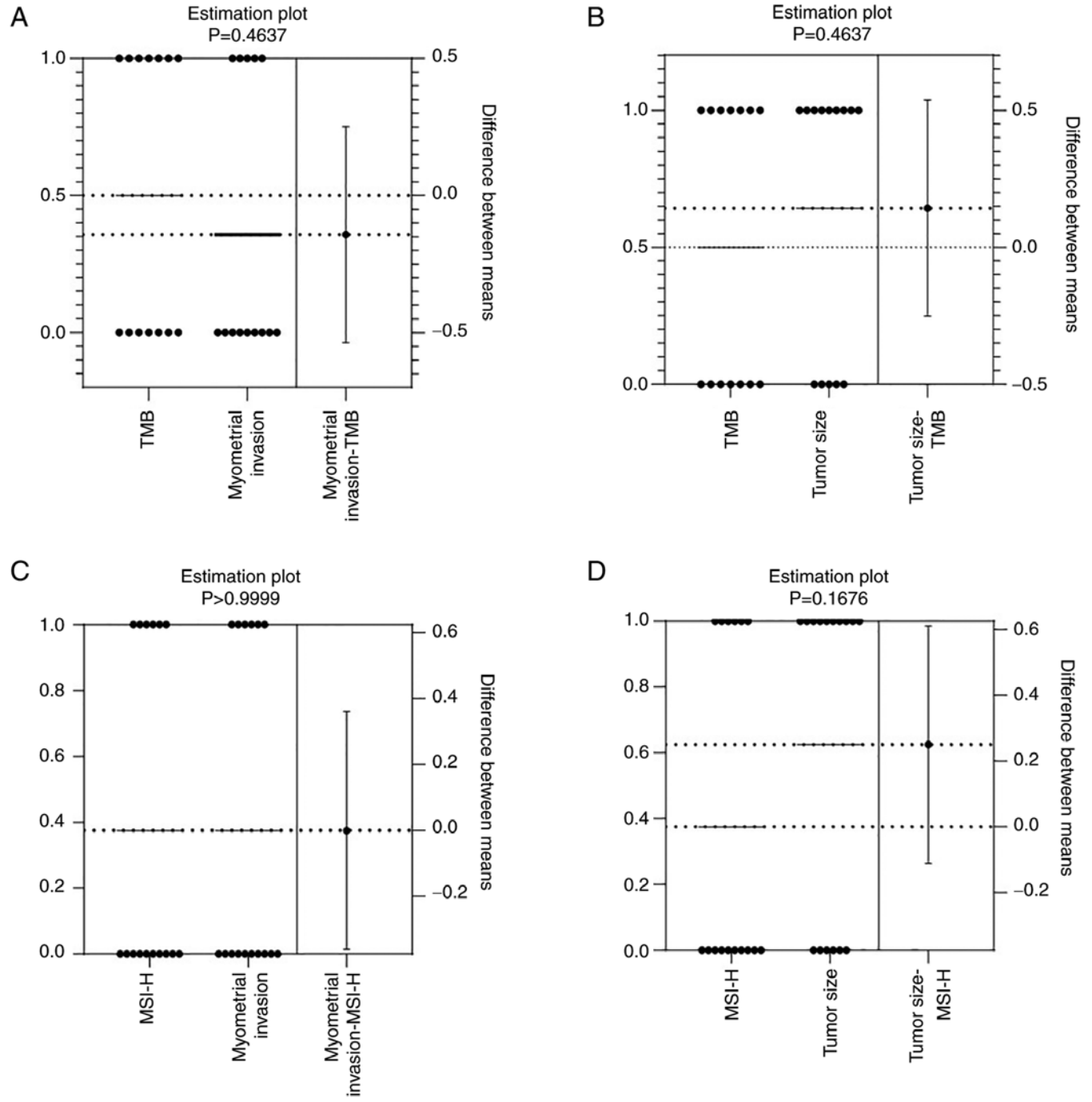

Figure 5. Correlation analysis of TMB and MSI-H status with invasiveness and tumor volume. (A) Correlation analysis of TMB and muscle infiltration (n=14). (B) Correlation analysis of TMB and tumor volume (n=14). (C) Correlation analysis of MSI-H status and muscle infiltration (n=16). (D) Correlation analysis of MSI-H status and tumor volume (n=16). MSI-H, microsatellite instability-high; TMB, tumor mutational burden.

Discussion

In the bloodstream of patients with solid tumors, the ratio of CTCs to white blood cells has been reported to be $1:10^6$-$1:10^7$, thus these cells are considered quite rare. Even so, the prognostic role of CTCs has been clearly demonstrated in numerous types of cancer (45). Magbanua *et al* (46) reported that the CTC trajectory pattern over the course of treatment was a good predictor of progression-free survival (PFS) and overall survival (OS). CTC counts of ≥2 and 5 per 7.5 ml have also been shown to be associated with reduced PFS and OS in patients with non-small cell lung cancer (47). Chen *et al* (48) reported that the CTC test accurately identified patients who were at a high risk for prostate cancer, allowing for the early intervention and effective treatment of patients. There are a number of types of sorting methods for CTCs, including the dielectrophoretic DLD method (49), the DEPArray™ system (50), emerging microfluidic technologies (51), dielectrophoretic enrichment (52) and the negative-selection enrichment method (48,53,54).

In the present study, CTCs were detected using the CytoBot® 2000 system, which works based on microscale meshes with a

nanofunctionalized coating that enables the efficient capture of CTCs (55). The results revealed that the positive rate for CTCs in patients with EC was 80.43%. The AUC value between EC and NO-EC groups was 0.8872, indicating that CTC detection had a good screening performance on EC.

lncRNAs are a class of RNA transcripts that are >200 nucleotides long (56). It has been reported that some lncRNAs have specific effects on tumor screening. For example, risk scores have been obtained for lncRNAs RP4-792G4.2 and RP11-325122.2 in glioblastoma, and their scores can be used for risk assessment (57). Additionally, RP11-54H7.4 is a possible prognostic target for tongue squamous cell carcinoma (58). Furthermore, the performance of the three-lncRNA signature comprising RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 has been reported to be higher in EC than in paracancerous tissue (24). On the basis of existing studies, the present study explored whether combining more indicators could improve the performance of a diagnostic model for EC.

In the present study, the levels RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 were measured in tumor tissues (n=35) and matched paracancerous tissues (n=35). However, these three indicators did not significantly distinguish tumor tissue from paracancerous tissue (P=0.2730, 0.0517 and 0.5180). The present study further evaluated whether these three indicators were effective in distinguishing between the EC (n=35) and NO-EC (n=14) groups. Notably, the performance of these indicators in differentiating the EC group (n=35) from the NO-EC group (n=14) was good (P=0.0002, 0.0001 and P<0.0001, respectively). Therefore, the lncRNAs RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6 may be suitable for distinguishing between the EC and NO-EC groups.

Qi *et al* (32) reported that the *CDO1*/*CELF4* dual-gene methylation assay had high sensitivity and specificity for AH and EC. Similarly, Krasnyi *et al* (42) reported that *CDO1* and *CDH13* gene methylation could predict early EC treatment outcomes. In the present study, methylated *CDO1* and *CELF4* were used to distinguish the EC group from the NO-EC group; however, the AUC values were only 0.6000 and 0.5286, respectively. Notably, when methylated *CDO1* and *CELF4* were added into the CTCs and lncRNAs panel, these two indicators could not improve the screening performance. The reason for this result may be only two methylated genes (*CDO1* and *CELF4*) were assessed. In addition, due to limited cell samples, the present study could not simultaneously conduct a number of molecular biology experiments. In the future, more gene indicators could be added and next generation sequencing may be used to improve EC screening performance.

MSI-H status and TMB are cancer-related conditions (43,44). The present study investigated whether these two indicators were related to the depth of muscle infiltration or EC tumor volume; however, the results revealed no significant correlation.

In conclusion, in the differentiation between EC and NO-EC groups, the performance of the combined model comprising CTCs and three lncRNAs (RP4-616B8.5, RP11-389G6.3 and CTD-2377D24.6) was promising.

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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

HD, YC and JY contributed to the study design, data analysis and writing of the manuscript. BL, JW and XZ contributed to the study design and writing of the manuscript. SX and HC contributed to CTC detection and statistics. JY and XZ provided the CTC detection instruments and chips. JM and LF contributed to experimental system verification and DNA methylation detection. JZ contributed to patient clinical information arrangement and data analysis. FS and HZ contributed to sample collection, lncRNA qPCR detection and statistics. HD provided funding. HD and JY confirm the authenticity of all the raw data. 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 The First Affiliated Hospital of Soochow University (approval no. 2021.351). The enrolled patients provided written informed consent before participation in this study, with permission for sample collection and analysis.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Li W, Xu Y, Zeng X, Tan J, Wang Y, Wu H, Li M and Yi C: Etiological relationship between lipid metabolism and endometrial carcinoma. Lipids Health Dis 22: 116, 2023.
2. Kokts-Porietis RL, Elmrayed S, Brenner DR and Friedenreich CM: Obesity and mortality among endometrial cancer survivors: A systematic review and meta-analysis. Obes Rev 22: e13337, 2021.
3. Wang X, Glubb DM and O'Mara TA: Dietary factors and endometrial cancer risk: A mendelian randomization study. Nutrients 15: 603, 2023.
4. Makker V, MacKay H, Ray-Coquard I, Levine DA, Westin SN, Aoki D and Oaknin A: Endometrial cancer. Nat Rev Dis Primers 7: 88, 2021.
5. Sheikh MA, Althouse AD, Freese KE, Soisson S, Edwards RP, Welburn S, Sukumvanich P, Comerci J, Kelley J, LaPorte RE and Linkov F: USA endometrial cancer projections to 2030: Should we be concerned? Future Oncol 10: 2561-2568, 2014.
6. Van Wijk F, Huikeshoven F, Abdulkadir L, Ewing P and Burger C: Stage III and IV endometrial cancer: A 20-year review of patients. Int J Gynecol Cancer 16: 1648-1655, 2006.

7. Vermij L, Jobsen JJ, León-Castillo A, Brinkhuis M, Roothaan S, Powell ME, de Boer SM, Khaw P, Mileshkin LR, Fyles A, *et al*: Prognostic refinement of NSMP high-risk endometrial cancers using oestrogen receptor immunohistochemistry. Br J Cancer 128: 1360-1368, 2023.
8. Crosbie EJ, Kitson SJ, McAlpine JN, Mukhopadhyay A, Powell ME and Singh N: Endometrial cancer. Lancet 399: 1412-1428, 2022.
9. Tian Y and Luo H: Diagnostic accuracy of transvaginal ultrasound examination for local staging of cervical cancer: A systematic review and meta-analysis. Med Ultrason 24: 348-355, 2022.
10. Elmstrøm-Christensen LB and Lauszus FF: Diagnostic delay of gynaecological cancer in women with postmenopausal bleeding. Dan Med J 69: A09210744, 2022.
11. Oaknin A, Bosse TJ, Creutzberg CL, Giornelli G, Harter P, Joly F, Lorusso D, Marth C, Makker V, Mirza MR, *et al*: Endometrial cancer: ESMO clinical practice guideline for diagnosis, treatment and follow-up. Ann Oncol 33: 860-877, 2022.
12. Tronconi F, Nero C, Giudice E, Salutari V, Musacchio L, Ricci C, Carbone MV, Ghizzoni V, Perri MT, Camarda F, *et al*: Advanced and recurrent endometrial cancer: State of the art and future perspectives. Crit Rev Oncol Hematol 180: 103851, 2022.
13. Vermij L, Smit V, Nout R and Bosse T: Incorporation of molecular characteristics into endometrial cancer management. Histopathology 76: 52-63, 2020.
14. Kasius JC, Pijnenborg JMA, Lindemann K, Forsse D, van Zwol J, Kristensen GB, Krakstad C, Werner HMJ and Amant F: Risk stratification of endometrial cancer patients: FIGO stage, biomarkers and molecular classification. Cancers (Basel) 13: 5848, 2021.
15. Kiss I, Kolostova K, Pawlak I and Bobek V: Circulating tumor cells in gynaecological malignancies. J BUON 25: 40-50, 2020.
16. Hu X, Zang X and Lv Y: Detection of circulating tumor cells: Advances and critical concerns. Oncol Lett 21: 422, 2021.
17. Castro-Giner F and Aceto N: Tracking cancer progression: From circulating tumor cells to metastasis. Genome Med 12: 31, 2020.
18. Law KS, Huang CE and Chen SW: Detection of circulating tumor cell-related markers in gynecologic cancer using microfluidic devices: A pilot study. Int J Mol Sci 24: 2300, 2023.
19. Lin W, Zhou Q, Wang CQ, Zhu L, Bi C, Zhang S, Wang X and Jin H: LncRNAs regulate metabolism in cancer. Int J Biol Sci 16: 1194-1206, 2020.
20. Park EG, Pyo SJ, Cui Y, Yoon SH and Nam JW: Tumor immune microenvironment lncRNAs. Brief Bioinform 23: bbab504, 2022.
21. Tan YT, Lin JF, Li T, Li JJ, Xu RH and Ju HQ: LncRNA-mediated posttranslational modifications and reprogramming of energy metabolism in cancer. Cancer Commun (Lond) 41: 109-120, 2021.
22. Xing C, Sun SG, Yue ZQ and Bai F: Role of lncRNA LUCAT1 in cancer. Biomed Pharmacother 134: 111158, 2021.
23. Zhang G, Sun J and Zhang X: A novel cuproptosis-related LncRNA signature to predict prognosis in hepatocellular carcinoma. Sci Rep 12: 11325, 2022.
24. Ding H, Jiang F, Deng L, Wang J, Wang P, Ji M, Li J, Shi W, Pei Y, Li J, *et al*: Prediction of clinical outcome in endometrial carcinoma based on a 3-lncRNA signature. Front Cell Dev Biol 9: 814456, 2021.
25. Xin W, Gao X, Zhao S, Zhao P, Yu H, Wu Q and Hua K: LncRNA RP11-395G23.3 suppresses the endometrial cancer progression via regulating microRNA-205-5p/PTEN axis. Am J Transl Res 12: 4422-4433, 2020.
26. Esteller M: Aberrant DNA methylation as a cancer-inducing mechanism. Annu Rev Pharmacol Toxicol 45: 629-656, 2005.
27. Nishiyama A and Nakanishi M: Navigating the DNA methylation landscape of cancer. Trends Genet 37: 1012-1027, 2021.
28. Wang Q, Xiong F, Wu G, Liu W, Chen J, Wang B and Chen Y: Gene body methylation in cancer: Molecular mechanisms and clinical applications. Clin Epigenetics 14: 154, 2022.
29. Papanicolau-Sengos A and Aldape K: DNA methylation profiling: An emerging paradigm for cancer diagnosis. Annu Rev Pathol 17: 295-321, 2022.
30. Jamshidi A, Liu MC, Klein EA, Venn O, Hubbell E, Beausang JF, Gross S, Melton C, Fields AP, Liu Q, *et al*: Evaluation of cell-free DNA approaches for multi-cancer early detection. Cancer Cell 40: 1537-1549.e12, 2022.
31. Caplakova V, Babusikova E, Blahovcova E, Balharek T, Zelieskova M and Hatok J: DNA methylation machinery in the endometrium and endometrial cancer. Anticancer Res 36: 4407-4420, 2016.
32. Qi B, Sun Y, Lv Y, Hu P, Ma Y, Gao W, Li S, Zhang X, Jin X, Liou Y, *et al*: Hypermethylated CDO1 and CELF4 in cytological specimens as triage strategy biomarkers in endometrial malignant lesions. Front Oncol 13: 1289366, 2023.
33. Wang L, Dong L, Xu J, Guo L, Wang Y, Wan K, Jing W, Zhao L, Feng X, Zhang K, *et al*: Hypermethylated CDO1 and ZNF454 in cytological specimens as screening biomarkers for endometrial cancer. Front Oncol 12: 714663, 2022.
34. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
35. Lawrence R, Watters M, Davies CR, Pantel K and Lu YJ: Circulating tumour cells for early detection of clinically relevant cancer. Nat Rev Clin Oncol 20: 487-500, 2023.
36. Yao H, Wen L, Li Z and Xia C: Analysis of diagnostic value of CTC and CTDNA in early lung cancer. Cell Mol Biol (Noisy-le-grand) 69: 57-62, 2023.
37. Francini S, Duraes M, Rathat G, Macioce V, Mollevi C, Pages L, Ferrer C, Cayrefourcq L and Alix-Panabières C: Circulating tumor cell detection by liquid biopsy during early-stage endometrial cancer surgery: A pilot study. Biomolecules 13: 428, 2023.
38. Li Y, Fan Z, Meng Y, Liu S and Zhan H: Blood-based DNA methylation signatures in cancer: A systematic review. Biochim Biophys Acta Mol Basis Dis 1869: 166583, 2023.
39. Harada H, Hosoda K, Moriya H, Mieno H, Ema A, Ushiku H, Washio M, Nishizawa N, Ishii S, Yokota K, *et al*: Cancer-specific promoter DNA methylation of cysteine dioxygenase type 1 (CDO1) gene as an important prognostic biomarker of gastric cancer. PLoS One 14: e0214872, 2019.
40. Kong LH, Xiao XP, Wan R, Chao XP, Chen XJ, Wang J, Wu HW and Li L: The role of DNA methylation in the screening of endometrial cancer in postmenopausal women. Zhonghua Yi Xue Za Zhi 103: 907-912, 2023 (In Chinese).
41. Huang RL, Su PH, Liao YP, Wu TI, Hsu YT, Lin WY, Wang HC, Weng YC, Ou YC, Huang TH and Lai HC: Integrated epigenomics analysis reveals a DNA methylation panel for endometrial cancer detection using cervical scrapings. Clin Cancer Res 23: 263-272, 2017.
42. Krasnyi AM, Gadzhieva LT, Kokoeva DN, Kosenko MG, Yarotskaya EL, Pavlovich SV, Ashrafyan LA and Sukhikh GT: Analysis of CDO1, PITX2, and CDH13 gene methylation in early endometrial cancer for prediction of medical treatment outcomes. Int J Mol Sci 25: 4892, 2024.
43. Salem ME, Bodor JN, Puccini A, Xiu J, Goldberg RM, Grothey A, Korn WM, Shields AF, Worrilow WM, Kim ES, *et al*: Relationship between MLH1, PMS2, MSH2 and MSH6 gene-specific alterations and tumor mutational burden in 1057 microsatellite instability-high solid tumors. Int J Cancer 147: 2948-2956, 2020.
44. Kawaguchi M, Banno K, Yanokura M, Kobayashi Y, Kishimi A, Ogawa S, Kisu I, Nomura H, Hirasawa A, Susumu N and Aoki D: Analysis of candidate target genes for mononucleotide repeat mutation in microsatellite instability-high (MSI-H) endometrial cancer. Int J Oncol 35: 977-982, 2009.
45. Vasseur A, Kiavue N, Bidard FC, Pierga JY and Cabel L: Clinical utility of circulating tumor cells: An update. Mol Oncol 15: 1647-1666, 2021.
46. Magbanua MJM, Hendrix LH, Hyslop T, Barry WT, Winer EP, Hudis C, Toppmeyer D, Carey LA, Partridge AH, Pierga JY, *et al*: Serial analysis of circulating tumor cells in metastatic breast cancer receiving first-line chemotherapy. J Natl Cancer Inst 113: 443-452, 2021.
47. Krebs MG, Sloane R, Priest L, Lancashire L, Hou JM, Greystoke A, Ward TH, Ferraldeschi R, Hughes A, Clack G, *et al*: Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. J Clin Oncol 29: 1556-1563, 2011.
48. Chen J, Xie T, Yang J, Lin X, Huang L, Su S and Deng J: Feasibility study of expressing epcam + /vimentin + CTC in prostate cancer diagnosis. J Cancer Res Clin Oncol 149: 8699-8709, 2023.
49. Rahmati M and Chen X: Separation of circulating tumor cells from blood using dielectrophoretic DLD manipulation. Biomed Microdevices 23: 49, 2021.
50. Di Trapani M, Manaresi N and Medoro G: DEPArray™ system: An automatic image-based sorter for isolation of pure circulating tumor cells. Cytometry A 93: 1260-1266, 2018.
51. Wei X, Chen K, Guo S, Liu W and Zhao XZ: Emerging microfluidic technologies for the detection of circulating tumor cells and fetal nucleated red blood cells. ACS Appl Bio Mater 4: 1140-1155, 2021.

52. S Iliescu F, Sim WJ, Heidari H, P Poenar D, Miao J, Taylor HK and Iliescu C: Highlighting the uniqueness in dielectrophoretic enrichment of circulating tumor cells. Electrophoresis 40: 1457-1477, 2019.
53. Burr R, Edd JF, Chirn B, Mishra A, Haber DA, Toner M and Maheswaran S: Negative-selection enrichment of circulating tumor cells from peripheral blood using the microfluidic CTC-iChip. Methods Mol Biol 2471: 309-321, 2022.
54. Andree KC, van Dalum G and Terstappen LW: Challenges in circulating tumor cell detection by the CellSearch system. Mol Oncol 10: 395-407, 2016.
55. Wang J, Dallmann R, Lu R, Yan J and Charmet J: Flow rate-independent multiscale liquid biopsy for precision oncology. ACS Sens 8: 1200-1210, 2023.
56. Chi Y, Wang D, Wang J, Yu W and Yang J: Long non-coding RNA in the pathogenesis of cancers. Cells 8: 1015, 2019.
57. Paul Y, Thomas S, Patil V, Kumar N, Mondal B, Hegde AS, Arivazhagan A, Santosh V, Mahalingam K and Somasundaram K: Genetic landscape of long noncoding RNA (lncRNAs) in glioblastoma: Identification of complex lncRNA regulatory networks and clinically relevant lncRNAs in glioblastoma. Oncotarget 9: 29548-29564, 2018.
58. Zhang M, Chen Z, Zhang S, Wu L, Jie Y, Liao Y, Huang Y, Chen J and Shi B: Analysis of differentially expressed long non-coding RNAs and the associated TF-mRNA network in tongue squamous cell carcinoma. Front Oncol 10: 1421, 2020.