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RESEARCH ARTICLE

Clinical significance of IL-37 serum level and polymorphism in patients with endometrial cancer

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ABSTRACT. *Background:* Endometrial cancer (EC) is recognized as the second most common type of cancer among women. Interleukin-37 (IL-37) is a recently discovered member of the IL-1 cytokine family characterized by its anti-inflammatory properties, which are believed to have both anti-tumour and tumorigenic effects. However, the precise role of IL-37 in the development of EC remains largely unknown. *Objective:* In the current study, we aimed to explore genotype and allele frequencies of the IL-37 gene (rs4241122) and measure IL-37 protein levels in patients with EC, with a view to determining the clinical significance in these patients. *Methods:* A total of 105 patients with confirmed EC and 105 healthy controls, aged 31-73, participated in the study. IL-37 serum levels were investigated using an ELISA method, while the frequency of genotypes and alleles of the IL-37 gene was determined using the ARMS-PCR method. *Results:* The findings demonstrate a significant increase in IL-37 serum levels in EC patients compared to controls ($p<0.0001$). Moreover, higher levels of IL-37 were strongly associated with unfavourable indices, such as EC grade III, poorly differentiated tumours, and regional spread of tumour cells ($p<0.05$). In contrast, genotyping of the IL-37 gene revealed no significant difference between the two groups, and there was no association between IL-37 genotype and IL-37 protein level or clinicopathological characteristics ($p>0.05$). *Conclusion:* The results of this study suggest that elevated serum levels of may contribute to tumour progression, probably through its immune suppressive activity. Clinically, IL-37 may serve as a promising factor and/or therapeutic target for EC management, although, further studies are warranted.

Key words: endometrial cancer; IL-37; polymorphism; ELISA

Endometrial cancer (EC) is considered the most prevalent gynaecological cancer and the second most common female cancer in developed countries [1, 2]. The estimated number of new cases diagnosed with EC, along with its mortality rate, has led to EC being described as one of the most pernicious gynaecological malignancies [3]. However, advances in disease management strategies, such as early diagnosis and novel treatment protocols, have led to a favourable increase in overall survival rates, with the five-year overall survival rate exceeding 81% [4, 5].

A plethora of evidence suggests that obesity-causing high-risk lifestyles, such as an inappropriate diet, body mass index (BMI) ≥ 30, and high waist-to-hip ratio, as well as a history of polycystic ovary syndrome, may be risk factors for EC incidence in premenopausal women [6, 7]. In addition to obesity, smoking and diabetes are considered risk factors for death caused by EC [8]. On the other hand, the vast majority of patients with EC are elderly women at postmenopausal age, which has led to the assumption that age is another main risk factor in the aetiology of uterine cancers [9]. Interestingly, both aging and obesity share a commonality in their disruption of the immune system. For instance, obesity is described as a chronic inflammatory state that causes the elevated release of pro-inflammatory cytokines and CRP. Therefore, altered immune responses have been identified as another risk factor in the aetiology of EC [10]. However, the exact molecular mechanism of immune system involvement in EC aetiology is not yet understood, and continuous efforts are being made to clarify this.

IL-37, a recently discovered member of the IL-1 family cytokine, consists of five different splice variants, termed IL-37a, IL-37b, IL-37c, IL-37d, and IL-37e. The 3.617-kb length gene, encoding human IL-37, is located on chromosome 2 [11, 12]. IL-37 is described as an anti-inflammatory factor that is believed to play a suppressive role in innate immunity and inflammatory responses

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[12]. Although the biological role of IL-37 in cancer is largely unknown, a multitude of recent studies have elucidated its antitumour and/or pre-tumoural functions in diverse cancer types. These studies have also unveiled several genetic and biochemical mechanisms involved in these functions [13-16]. However, the role of IL-37 and its different variants in the pathogenesis of EC, as well as its potential as a diagnostic biomarker and immunotherapeutic target, has not been fully determined yet.

To the best of the authors' knowledge, the serum level of IL-37 and its genetic variants have not been previously fully investigated in patients with EC, and the precise role of IL-37 in EC remains to be determined. Therefore, the current study aimed to evaluate IL-37 at both the protein and gene level in patients with EC, with a view to determining the clinical significance of our findings. In this regard, in the current study, we measured the level of IL-37 in the sera of patients with EC, relative to a control group, and further determined the frequency of genotypes and alleles in the IL-37 gene (rs4241122) in these patients. The data was then analysed to determine possible associations with the clinicopathological characteristics of patients with EC.

MATERIALS AND METHODS

Ethical approvement

The current case-control study was conducted following the ethical standards outlined in the 1975 Declaration of Helsinki, as revised in 2008. Furthermore, this study obtained approval from the ethics committee of the Sirjan School of Medical Sciences (IR.SIRUMS.REC.1400.032).

Study population and sample collection

After distributing the participants into two groups, patients (cases) and healthy individuals (controls), an informed consent form was signed by both groups of participants. The case group consisted of recently diagnosed EC patients, aged 32-86 (mean age: 56.81 ± 10.81), who had been referred to Zeinabiyyeh and Faghihi Hospitals in Shiraz, Iran. The inclusion criterion for the case group was confirmation of the disease through both clinical and pathological examinations. Exclusion criteria for the cases included a history of other types of malignancy, autoimmune disorders, immune deficiency disorders, prior surgery, and any evidence of infectious diseases within the last month. The control group, aged 32-85 (mean age: 55.21 ± 13.80), consisted of healthy individuals from southern Iran who were matched to the patient group in terms of age. They had no history of cancer or autoimmune diseases and had not experienced any infectious or inflammatory diseases within the past month. Control subjects were included in the study after meeting the aforementioned criteria, as confirmed by a gynaecologist.

Subsequently, approximately 5 mL of venous blood was collected from all participants, comprising 105 patients and 105 controls. The blood samples were collected in tubes containing EDTA anticoagulant (10% solution) for genotyping analyses. In addition, 30 patients with EC, aged 31-73 (mean age: 52.20 ± 11.85) and 33 healthy controls, aged 31-73 (mean age: 52.18 ± 11.21), were randomly selected for further protein analysis, and their venous blood was collected in tubes without EDTA anticoagulant. The patients' blood samples were obtained before any medical interventions, such as surgery or chemo/radiotherapy. Subsequently, the blood samples were centrifuged at 2500 × g for 10 minutes at 4 °C to separate the serum. The serum was then aliquoted and preserved at -80 °C for subsequent analysis. Additionally, genomic DNA was extracted from leukocytes using a previously described method, known as the "standard salting-out procedure", with minor modifications [17].

In addition to collecting samples from patients, demographic information and pathological conditions of the cases were documented. This information included age at the time of diagnosis, menstrual age, menopausal age, type of tumour, degree of differentiation, FIGO grade, tumour size, myometrial invasion, menopause status, and location of the tumour.

Measurement of IL-37 serum level in EC patients and healthy subjects

A commercially available human IL-37 enzyme-linked immunosorbent assay (ELISA) kit was obtained from Shanghai Crystal Day Biotech Co., Ltd for the purpose of measuring the levels of IL-37 in serum. The ELISA kit provided had an assay range spanning from 7 pg/mL to 400 pg/mL, with a sensitivity of 4.5 pg/mL.

Genotype analysis

The IL-37 gene, together with eight members of IL-1 family genes, form a cytokine gene cluster on chromosome 2. Five alternatively spliced transcript variants encoding distinct isoforms of IL-37, namely IL-37a, IL-37b, IL-37c, IL-37d, and IL-37e, have been reported. Additionally, five single-nucleotide polymorphisms (SNPs) spanning the entire region have been identified for IL-37, which are reported to increase genetic susceptibility to different types of diseases [18]. These SNPs are rs2723186, rs3811046, rs4241122, rs4364030, and rs4392270. Among them, rs4241122 has shown a fixed minor allele frequency (MAF) greater than 0.05 in Chinese (MAF = 0.146) and Iranian (MAF = 0.2) populations [18, 19]. Consequently, this SNP was selected to investigate the association between IL-37 genetic variation and susceptibility to EC. For this purpose, the DNA was extracted from the blood samples. Subsequently, 500 ng of genomic DNA was combined with 5 µL of master mix (2×) (Parstous, Tehran, Iran), 2.5 µL of distilled water, and 0.5 µL (10 pM) of primers (Metabion, Germany). Next, the amplification refractory mutation system polymerase chain reaction (ARMS-PCR) method was performed. The online Primer3 software version 0.4.0 was used for designing the common primer for each SNP and allele-specific ARMS primers. The PCR conditions are summarized in *table 1*. The PCR products, along with a 50-1500 bp ladder, were separated by gel electrophoresis on a 2% agarose gel stained with a safe stain. Finally, the products were visualized using an ultraviolet transilluminator. *Figure 1* illustrates the genotyping of the IL-37 gene (rs4241122) using the ARMS-PCR method.

Table 1
The sequences of primers and PCR conditions used in the study.

Genes	Primers	Sequences	Reaction products	PCR condition		
				Stage	Time	Temp
IL-37 (rs4241122)	*FI*	5′ -CAGGCTCTAGACTGACTCCA-3′	Main band: 355bp	Initial denaturation	5 min	95°C
	FII	5c- CAGGCTCTAGACTGACTCCG-3′		Denaturation	35 sec	94°C
	R	5′ -TCAAACTATCAACATCAAGGCACA-3′	Internal control band: 110bp	Annealing	**30 sec**	66°C
B2Globin	*F*	5′-ACACAACTGTGTTCACTAGC-3′		Extension	30 sec	72°C
	R	5-′CAACTTCATCCACGTTCACC-3′		Ultimate extension	5 min	72°C

B2Globolin was used as the internal control. FI: primer forward 1; FII: primer forward 2; R: primer reverse; Temp: temperature.

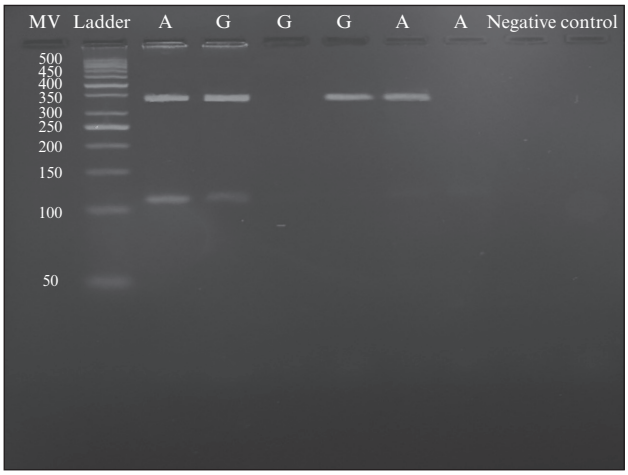

Figure 1
Genotyping of IL-37 gene (rs4241122) based on the ARMS-PCR method. The first lane represents a 50-1500 bp ladder. Lanes 2, 3: the presence of two predominant bands (both corresponding to 355 bp) and two internal control bands (both corresponding to 110 bp) indicates genotype AG. Lanes 4, 5: the presence of one of the predominant bands (355 bp) together with an internal control band (110 bp) indicates the GG genotype. Lanes 6, 7: the presence of one of the predominant bands (355 bp) together with an internal control band (110 bp) indicates the AA genotype. The last lanes correspond to negative controls.

Statistical analysis

Statistical analysis was performed using SPSS software (version 24) and GraphPad Prism (version 8.0). The normal distribution of variables was determined using the Kolmogorov-Smirnov or Shapiro-Wilk's tests. The deviation of SNPs from the Hardy-Weinberg Equilibrium was evaluated using the Arlequin software package based on the comparison of the frequencies of observed and expected genotypes. The comparative analysis between cases and controls, as well as association analysis, were performed using the two-tailed Pearson $\chi 2$-test, the Mann-Whitney U, and the Kruskal-Wallis H test. *P* value <0.05 was considered to reveal a significant result.

RESULTS

Clinicopathological characteristics of EC patients

The present study investigated the characteristics of 105 patients with EC, including age (age at diagnosis, age at menstruation, and age at menopause), tumour grade (grade I/II, grade III/IV), degree of tumour differentiation (well differentiated, moderately differentiated, poorly differentiated), FIGO grade (tumour confined to the corpus uteri, invasive cervical stromal tumour, local and/or regional spread of the tumour), tumour size (less than 2 cm, more than 2 cm), myometrial invasion (up to 50, under 50), and menopausal status (menopausal or not). As shown in *table 2*, the majority of EC patients were in stage I/II (70.5% *vs* 29.5%, with EC grade III), had well-differentiated tumours (54.3% *vs* 23.8% moderately differentiated and 21.9% poorly differentiated), had tumours confined to the corpus uteri (60% *vs* 23.8% with invasive cervical stromal tumour and 16.2% local and/or regional spread of the tumour), had tumour size greater than 2 cm (70.5% *vs* 27.6% with less than 2 cm), had myometrial invasion under 50 (73.3% *vs* 20% with up to 50), and were menopausal (81.9% *vs* 18.1% not menopausal).

Comparison of IL-37 serum level between EC patients and healthy controls

The current study utilized a commercial kit to measure and compare the serum levels of IL-37. As depicted in *figure 2*, the results revealed a significant difference ($p<0.0001$) in the serum levels of IL-37 when comparing patients with EC (141.81 ± 10.63 pg/mL) to matched healthy controls (33.23 ± 2.29 pg/mL).

Genotypes and allele frequencies of the IL-37 gene (rs4241122) in EC patients

Genotyping distribution of IL-37 rs4241122 SNP in patients with EC and healthy subjects revealed no deviation from the Hardy-Weinberg equilibrium. As shown in *table 3*, the genotype ($p=0.30$) and allele ($p=0.24$) distribution of rs4241122 G>A between EC patients and healthy controls was not significantly different.

Correlation between serum level of IL-37 and rs4241122 SNP

Linear regression analysis was performed to establish any association between rs4241122 SNP and serum level of IL-37 in patients with EC. The obtained results demonstrated that there was no significant correlation between rs4241122 SNP and IL-37 serum level in EC patients ($p>0.05$).

Table 2
Demographic and clinicopathological characteristics of the 105 patients with EC in the current case-control study.

Demographic and clinicopathological characteristics of patients (N = 105)		Collected data
Age	*Age at diagnosis*	56.81±10.81
	Menstrual age	12.82± 1.52
	Menopausal age	49.39±8.70
Tumour type	*EC grade I/II*	74 (70.5%)
	EC grade III (clear cell undifferentiated papillary serous)	31 (29.5%)
Tumour differentiation	*Well differentiated*	57 (54.3%)
	Moderately differentiated	25 (23.8%)
	Poorly differentiated	23 (21.9%)
FIGO grade	*Tumour confined to the corpus uteri*	63 (60%)
	Invasive cervical stromal tumour	25 (23.8%)
	Local and/or regional spread of the tumour	17 (16.2%)
Tumour size	*<2cm*	29 (27.6%)
	>2 cm	74 (70.5%)
	Missing	2 (1.9%)
Myometrial invasion	*Up to 50*	21 (20%)
	Under 50	77 (73.3%)
	Missing	7 (6.7%)
State of menopause	*Menopausal*	86 (81.9%)
	Not menopausal	19 (18.1%)
Location of tumour	*Endometrium*	105 (100%)

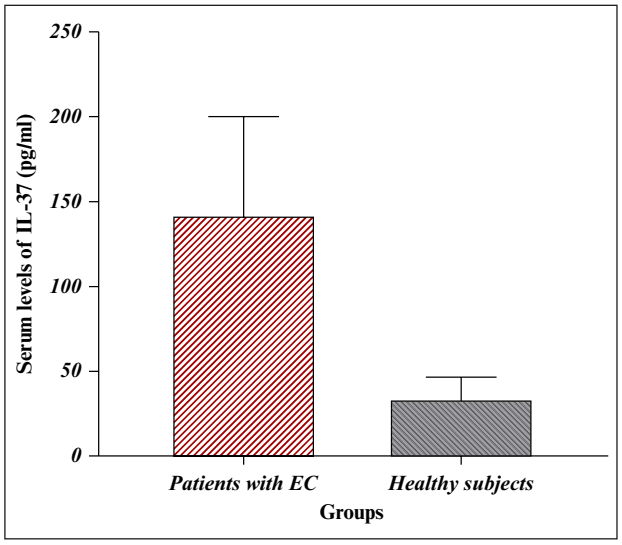

Figure 2
The serum level of IL-37 compared between patients with EC and healthy subjects. The level of IL-37 was remarkably higher in EC patients when compared to healthy individuals. $P<0.05$ was considered significant.

Correlation between IL-37 serum level or IL-37 rs4241122 SNP and clinicopathological characteristics

The serum level of IL-37 showed a significant association with tumour type (p=0.02), histological grade (p=0.03), FIGO grade (p=0.02), and menopausal status (p=0.004). Specifically, type III clear cell undifferentiated papillary serous, poorly differentiated tumours, local and/or regional spread of the tumour, and being menopausal were associated with significantly higher IL-37 serum levels in EC patients. The summarized data is presented in *table 4*. However, tumour size and myometrial invasion did not exhibit a significant association

Table 3
The frequency of genotypes and alleles resulting from IL-37 gene polymorphisms at position rs4241122 revealed no significant differences between EC patients and healthy subjects. $P<0.05$ was considered significant.

IL-37		Patients	Controls	*p* value
Genotype	AA	45(42.9%)	41(39.80%)	0.30
	AG	46(43.8%)	40(38.80%)	
	GG	14(13.3%)	22(21.40%)	
Allele	A	136(64.74%)	122(59.22%)	0.24
	G	74(35.24%)	84(40.78%)	

with serum level of IL-37 (p>0.05). Regarding the association analysis between IL-37 rs4241122 SNP and EC manifestations, no significant evidence of an association was found between genotype and tumour type (p=0.47), histological grade (p=0.086), FIGO grade (p=0.40), tumour size (p=0.33), myometrial invasion (p=0.60), or menopausal status (p=0.90).

DISCUSSION

The present study aimed to clarify the role of IL-37 as an anti-inflammatory cytokine in EC by determining serum levels, genotype, and alleles of this cytokine in patients with EC. Additionally, the study sought to investigate a possible association between IL-37 serum level, polymorphism of IL-37 (rs4241122) and clinicopathological characteristics.

The current findings reveal that serum levels of IL-37 were significantly higher in patients with EC compared to healthy controls. Furthermore, the study demonstrated that higher levels of IL-37 were associated with unfavourable indices of EC, such as grade III/IV (as opposed to grade I/II), poorly differentiated tumours (versus well-differentiated and moderately

Table 4
Association between IL-37 serum level and clinicopathological characteristics of patients with EC.

Clinicopathological manifestation		IL-37 level		
		Mean	SEM	*P* value
Tumour type	EC grade I/II	130.53	8.35	0.02*
	EC grade III	198.23	43.33	
Histological grade	Well differentiated	124.74	5.99	0.03*[1]
	Moderately differentiated	145.05	22.06	
	Poorly differentiated	241.31	62.71	
FIGO grade	Tumour confined to the corpus uteri	121.32	4.62	0.02*[2]
	Invasive cervical stromal tumour	149.24	22.02	
	Local and/or regional spread of the tumour	251.80	56.72	
Tumour size	< 2cm	120.15	8.21	0.30
	≧ 2cm	150.43	13.90	
Myometrial invasion	Up to 50	129.32	6.04	0.77
	Under 50	164.01	40.71	
Menopause	Yes	154.79	14.25	0.004**
	No	111.54	4.30	

Values are represented as mean ± SEM. *p<0.05, **p<0.01, based on the Kruskal-Wallis (K-W) and Man-Whitney (M-W) tests.

[1] Mann–Whitney U test; IL-37 serum levels were found to be significantly higher in poorly differentiated tumours than in well-differentiated (p=0.003) and moderately differentiated tumours (p=0.048).

[2]Mann-Whitney U test; IL-37 serum levels were found to be significantly higher in the local and/or regional spread of the tumour in comparison with tumour confined to the corpus uteri (p=0.001) and invasive cervical stromal tumour (p=0.048).

differentiated tumours), and local and/or regional spread of the tumour (as compared to tumours confined to the corpus uteri and tumours that invade the cervical stromal layer). In line with our results, previous studies have shown that patients with epithelial ovarian cancer exhibit significantly higher serum levels of IL-37 compared to age-matched healthy controls. Moreover, elevated IL-37 levels have previously been associated with unfavourable prognosis and indices of tumour progression, including tumour size, residual tumour size, FIGO stage, lymph node metastasis, and positive recurrence [20]. Accordingly, our study on bladder cancer revealed significantly higher levels of IL-37 in male patients and patients aged 70 or older. Additionally, bladder cancer patients with perineural invasion demonstrated a non-significant trend of higher serum IL-37 levels compared to those without bladder cancer, which may potentially be associated with a poor prognosis [21]. Additionally, our study on patients with brain tumours revealed a simultaneous elevation of IL-37 and IL-18 binding protein (IL-18BP) serum levels in low- and high-grade malignancies [22]. In this regard, it is assumed that by binding to the IL-18 receptor and IL-18BP, IL-37 may potentially suppress the biosynthesis of IL-18-dependent pro-inflammatory cytokines and inhibit innate and adaptive immune responses, hence it might promote brain tumour progression, as reported for renal ischaemic injury [23]. Similarly, it has been shown that IL-37 levels were significantly increased in colorectal cancer patients and negatively correlated with CD8+ T cell infiltration [24]. CD8+ lymphocytes and dendritic cells are considered the most powerful cells involved in anti-tumour responses [25, 26]. Furthermore, *in vitro* analysis demonstrated that IL-37b could inhibit T cell priming by suppressing dendritic cell maturation and producing T cell inhibitory cytokines [27]. Consistent with the confirmation of the immune suppressive and pre-tumoural functions of IL-37, blood samples from patients with melanoma were shown to have upregulated IL-37 mRNA levels. Additionally, IL-37 mRNA was found to be highly expressed in human CD4+ CD25+ regulatory T cells (Tregs), further suggesting its immunosuppressive activity [28, 29]. It has been documented that Treg cells can significantly suppress T cell responses and the activity of cytotoxic lymphocytes, thereby attenuating antitumour responses [30, 31]. As a result, IL-37 likely plays a pivotal role in suppressing antitumour immune responses, thereby promoting tumour growth. It can be assumed that the elevated levels of IL-37 in the sera of EC patients contribute to pre-tumour events that facilitate tumour growth.

Contrary to the findings of the present study, an *in vitro* study revealed that IL-37 was downregulated in endometrial carcinoma cells and did not affect the proliferation of these cells. However, IL-37 effectively suppressed the invasion of EC cells [32]. In addition, several studies have determined that IL-37 exhibits tumour suppressor properties against a variety of cancers, including non-small cell lung cancer [33], breast cancer [34], renal cell carcinoma [35], colon cancer [15], and hepatocellular carcinoma [36]. These effects are mediated through the modulation of intracellular signalling pathways. In addition, patients with oral squamous cell carcinoma, who have lower levels of IL-37, are significantly susceptible to developing advanced tumour stages and lymph node metastasis [37]. Furthermore, lower levels of IL-37 in pancreatic and non-small cell lung cancers have been associated with diminished disease-free survival and overall survival [38, 39]. These contradictions among different studies may be attributed to variations in the types of

cancer studied, as well as the diverse tumour microenvironment. Therefore, it is crucial to measure IL-37 levels in different types of cancer. Additionally, the tumour-specific expression of IL-37 and its dual role in tumours have been recognized. IL-37 is believed to have an anti-tumour function in the early stages of tumourigenesis by preventing the normal-to-cancer cell transition and exhibiting anti-inflammatory properties. However, in the advanced stages of cancer, IL-37 may contribute to malignancy by reinforcing the immunosuppressive properties of Treg cells and suppressing T cell priming through modulation of dendritic cell maturation and cytokine production [29, 40]. Therefore, the findings of the present study suggest the potential of IL-37 as a possible therapeutic target for EC patients. However, further studies with larger sample sizes are needed to confirm this hypothesis and evaluate the efficacy of IL-37 as a diagnostic/therapeutic biomarker and predictor of EC prognosis.

Furthermore, in the current study, no significant difference in the frequency of genotypes and alleles of IL-37 gene polymorphisms was identified between EC patients and healthy controls. Additionally, genotyping of IL-37 SNP revealed no significant association with IL-37 serum level or clinicopathological characteristics of EC patients. These findings may indicate that IL-37 gene polymorphism does not play a major role in EC patho-aetiology and emphasize the probable compensatory nature of increased levels IL-37 to confront cancer. Although IL-37 SNPs associated with inflammatory and infectious diseases, such as rheumatoid arthritis [41] and Helicobacter pylori infection [42], have been reported, conflicting evidence indicates a lack of association between IL-37 gene polymorphism and Behcet's disease [19, 43] or hepatitis B infection [44]. Therefore, further studies considering other IL-37 gene polymorphism positions, involving patients with different ancestries, are encouraged to validate the diagnostic and therapeutic potential of IL-37 in EC patients.

CONCLUSION

The results of the current study demonstrate that IL-37 is significantly increased in patients with EC and that these levels are significantly associated with unfavourable indices, which may serve as a promising factor for disease diagnosis, prognosis prediction, and targeted therapy. Nevertheless, further studies with a larger sample size are necessary to validate the findings.

DISCLOSURE

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Conflicts of interest: none.

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