Investigation of TGF-β1 gene variant and expression in a group of Iranian women with endometriosis

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Abstract

Purpose

Endometriosis is defined as a common gynecologic and inflammatory disease. Transforming growth factor-beta 1 *(TGF-β1)* gene and its protein level might play a role in the pathogenesis of endometriosis. The present study aimed for the first time to assess the associations between endometriosis risk and − 509 C/T (rs1800469) variant of the *TGF-β1* gene as well as TGF-β1 mRNA expression in eutopic endometrium tissue of patients with and without endometriosis among a group of Iranian women.

Methods

Genotyping was carried out in 100 endometriosis patients (cases) with confirmed histological diagnosis of endometriosis and 197 non-endometriosis subjects (controls). The expression level of TGF-β1 mRNA was determined using Real-Time PCR assay in 15 eutopic endometrium tissue of women with endometriosis and 15 healthy controls.

Results

There was a significant association for allele and genotype frequencies of rs1800469 variant and endometriosis i. No significant difference for TGF-β1 expression was observed between eutopic endometrium of patients and healthy group. Also, evaluation of TGF-β1expression across the menstrual cycle showed the same level of TGF-β1 among cases and controls subjects.

Conclusion

Our investigations indicated enough evidence for the effect of *TGF-β1* gene polymorphism on endometriosis risk in an Iranian population. Furthermore, we could not find any relations between TGF-β1 mRNA expression and susceptibility to endometriosis.

Introduction

Endometriosis is defined as a common gynecologic, estrogen-dependent, chronic, and inflammatory disease that is affecting around 6–10% of women in their reproductive period and is a major contributor to chronic pelvic pain, dysmenorrhoea, dyspareunia, and subfertility [1–3]. It is described by the existence of endometrial-like tissue outside the uterine cavity, mainly on the pelvic peritoneum, ovaries and rectovaginal area [4, 5]. The prevalence of endometriosis disease seems to be ~ 5% among women between 25 years and 35 years of age [6]. It is associated with primary or secondary infertility in 30% of the women [7]. Although, the pathogenesis of endometriosis is unknown, the development of this polygenic and multifactorial disease is influenced by a combination of genetic, immunologic, hormonal, and environmental factors [8, 9]. Up until now, the exact etiology of endometriosis has been not known while there is increasing evidence that *transforming growth factor-beta 1 (TGF-β1)* gene and its protein level might play a role in the pathogenesis of inflammatory disease, including endometriosis [10]. Because *TGF-β1* is a multifunctional cytokine that is involved in endometrial stromal cells proliferation and differentiation [11], angiogenesis [12], formation of adhesion [13] and immune response [10, 14].

Several lines of evidence represent that susceptibility to the development of endometriosis is somewhat influenced by genetic factors. Accordingly, with respect to the role of *TGF-β1* as a candidate gene in susceptibility to endometriosis, investigation of -509 C/T variant (rs1800469) of the *TGF-β1* gene, which is the main determinant of plasma TGF-β1 concentration, will be beneficial for determining the role of this genetic factor in development of endometriosis [9, 15]. Nonetheless, the role of the aforementioned polymorphism in risk of endometriosis has not been investigated in Iranian population so far. Therefore, the present study aimed to assess the associations between endometriosis and the − 509 C/T polymorphism of the *TGF-β1* gene as well as TGF-B1 mRNA expression among a group of Iranian women for the first time.

Method

The present study aimed at two points; first, analysis of the associations between the − 509 C/T (rs1800469) variant of the *TGF-β1* gene and endometriosis risk. Second, assessing the TGF-β1 mRNA expression in eutopic endometrium tissue of patients with and without

endometriosis.

Blood Sample

Blood samples were taken from a totally 297 subjects who had undergone laparoscopy or laparotomy for diagnosis or treatment of endometriosis to assess the association between *TGF-β1* -509 C/T genetic variant and endometriosis susceptibility.

The samples were enrolled from two hospitals (Firoozgar and Valiasr) between April 2013 and September 2015 and the diagnosis of endometriosis was based on surgical and histologic criteria.

The study group (case) was involved 100 Iranian patients with endometriosis with the histologically confirmed diagnosis of endometriosis after their operative findings, according to the presence of endometrial glands or stroma in the lesions.

Since endometriosis is estrogen-dependent, the control group was selected from women at reproductive age and before menopause with a definite diagnosis of the absence of endometriosis by a surgeon.

We included 197 healthy volunteers who underwent laparoscopy, and laparotomy due to tubal ligation or treatment of benign diseases such as ovarian cysts, myoma, hydrosalpinx, or other reasons.

Individuals with a history of malignancy, those at menopausal age, and patients who had received hormonal treatment for at least 6 months since sampling were excluded from the study.

Three peripheral blood samples (in EDTA containing vial) were assembled from all subjects (females with endometriosis and controls females) for genotyping at the time of enrollment and stored at -20 °c until DNA extraction.

Tissue Sample

For evaluating the TGF-β1 mRNA expression, samples of eutopic endometrium tissue were obtained by sharp curettage of the corpus of the uterine cavity of both 15 healthy controls and 15 women with endometriosis who had undergone a surgical resection between April 2013 and September 2015 at two Hospitals (Firoozgar and Valiasr) and was snap-frozen in liquid nitrogen and stored at -80°C until used.

Written informed consent and answer a questionnaire about demographics and clinical characteristics such as gravidity, parity, abortion, dysmenorrhea, menes, and bleeding were received from all the participants who were volunteers for blood and tissue sampling before they enrolled. Dysmenorrhea and bleeding were classified based on the amount of the pain (Normal, Severe, Not) and bleeding (Normal, Severe, Low) in the menstrual time. Regarding menes, subjects also were categorized as regular and irregular.

SNP genotyping

Genomic DNA from whole blood was isolated from all subjects using phenol protocol. Extracted DNA was labeled and stored at -20°C until use. In the isolated DNA, we identified − 509 C/T polymorphic site in the promoter region of the *TGF-ß1* gene with the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method. PCR primers were designed based on the Genbank reference sequence. The primers sequences that used for amplifying DNA were listed in the Table 1. PCR amplification was carried out in a final reaction volume of 20 µl final reaction containing 1µl template DNA, 10µl Red Master Mix (Amplicon), 0.5µl each primer and 8µl H_2O by a Biorad MJ mini (Singapore) device. PCR cycle was done under the following conditions: an initial denaturation step at 95°c for 5 minutes followed by 35 cycles of 30 seconds at 95°c, 30 seconds at 59°c; 35 seconds at 72°c, and a final elongation at 72°c for 10 minutes. The allelic variant − 509 C (a 2.5 µl PCR product C) can be digested with 0.25 µl restriction enzyme *Bsu36*I (New England Biolab) at 37°C overnight in a 10 µl reaction that produced one fragment of 419 bp for the CC genotype; three fragments of 419, 229, and 190 bp for the CT genotype; and two fragments of 229, and 190 bp for the TT genotype.

Table 1
Primers that used in PCR and RT-PCR assay

PCR	Primers
TGF-ß1	5'-CAGACTCTAGAGACTGTCAG-3'
-509 C/T	5'-GTCACCAGAGAAAGAGGAC-3'
(rs1800469)	
RT-PCR	Primers
TGF-ß1	5-CCATGAACTTTCTGCTGTCTT-3
	5-TCGATCGTTCTGTATCAGTCT-3
HPRT	5'-CCTGGCGTCGTGATTAGTGAT-3'
	5'- TTATGGACAGGACTGAACGTCT-3'
PCR, Polymerase chain reaction; RT-PCR, Reverse transcription polymerase chain reaction ; TGF-ß1, Transforming growth factor-beta 1; HPRT, Hypoxanthine-guanine phosphoribosyltransferase	

5 µl of digestion product and 7µl of Ready-Load 100-bp DNA Ladder (Invitrogen, Spain) were loaded into 3% agarose gel (Invitrogen, Spain) containing 1.4µl of sybregreen. The gel underwent electrophoresis at 120V, 100 mA for 30 min. The gel was visualized using a UV light (Wealtec, South Africa), and photographs of gels were taken after staining. We confirmed the genotypes identified with enzyme digestion by Sanger sequencing.

mRNA VEGF expression

Isolation of RNA and complementary DNA Synthesis

In brief, total RNA from frozen tissue was extracted using the fermentas kit (Qiagen Inc., Valencia, CA, USA), in accordance with the manufacturer's instructions. RNA concentration and purity were measured using NanoDrop 2000 (Thermo Scientific, USA) at a wavelength of 260 nm, and both mRNA and protein concentrations were measured at a wavelength of 280 nm. Two micrograms of total RNA were subjected to reverse transcription into the first-strand cDNA with a commercially available kit (Thermo Scientific, USA) according to the manufacturer's protocol. In the first step, 0.5µl primer (Random Hex) (100µm) and 3.5µl dH_2O was added to 2µl of total RNA and annealed at 65°c for 5 minutes. Then, 2µl primescript buffer (5x), 1µl dNTP, 0.5µl Revertaid and 0.5µl Ribolock was added in the last tube at 25°c for 5 minutes, 42°c for 1 hour and 70°c for 5 minutes. The cDNA was stored at -80°C until used.

RT-PCR

Transcribed products were subjected to PCR for *TGF-ß1* and hypoxanthine-guanine phosphoribosyltransferase *(HPRT)* in a 15µl final reaction volume. For a negative control, the cDNA template was omitted from the reaction. Amplification for *TGF-ß1* cDNA was started with 5 minutes denaturation at 95°c, followed by cycles of 30 seconds of denaturation at 94°c, 30 seconds of annealing at 53°c, and 5 minutes of extension at 72°c. The PCR profile for *HPRT* began with 95°c denaturation for 5 minutes, followed by cycles of 39 seconds of denaturation at 95°c, 40 seconds of annealing at 60°c, and 5 minutes of extension at 72°c.

The primers used for *TGF-ß1* and HPRT amplification were shown in the Table 1. Final PCR products were exposed to electrophoresis through at 2% agarose gel and stained with sybregreen. The size of reverse transcription polymerase chain reaction (RT-PCR) products for *TGF-ß1* and HPRT were 83 and 131 bp, respectively.

Real-Time PCR

Concisely, the real-time PCR amplification was accomplished in a total volume of 20µl, containing 6µl of cDNA sample, 9.8µl sybre, 0.2µl Rox, 0.6µl of each primer and 2.8 dH_2O.The reactions were done in duplicate by ABI System (Applied Biosystems, Foster City, CA). The real-time PCR program consisted of 10 minutes at 95°C, followed 5 seconds at 95°C, 34 seconds at 53, 15 seconds at 95°c, 1hour at 53 °c and 15 seconds at 95.

Statistical analysis

All statistical analysis was donning using the Stata software version 14.2.

Case and controls were compared using the Kruskal-Wallis test for the quantitative variables such as age, BMI, parity, gravidity, and abortion. Data were expressed as the mean ± standard deviation (SD).

Chi-squared test was used for comparison of qualitative values (categorical) including dysmenorrhea, menes, and bleeding, presented by number and percentage.

Differences of *TGF-ß1*genotype and allele frequencies between endometriosis patients and controls were compared using Logistic regression and an odds ratio (OR) with 95% confidence interval (CI) were used as a measure of the strength of association between genotypes, allele frequencies. The Mann–Whitney U test was used to evaluate the association of the mRNA TGF-B1 expression with endometriosis risk.

Deviations from Hardy-Weinberg equilibrium (HWE) in control group were assessed using Chi-squared test (X^2).

Probability values less than or equal to 0.05 were considered statistically significant.

Result

Characteristics of the patients and controls

Table 2 shows characteristics of the endometriosis patients and controls including age, BMI, gravidity, parity, abortion, dysmenorrhea, menes, and bleeding.

Table 2
Characteristics of endometriosis patients and controls women in a group of Iranian population

	Endometriosis N = 100	Normal N = 197	P-value
Age (years)			
Number Total = 296	N = 99	N = 197	0.90
	35.39 ± 7.56	35.50 ± 11.00	
BMI (kg/m^2)			< 0.01
Number Total = 285	90	192	
	25.47 ± 4.66	28.45 ± 4.15	
< 18.5	5 (5.5)	0 (0)	
18.5–24.9	44 (48.8)	42 (52)	
25-29.9	27 (30)	82 (42.7)	
> 30	14 (15.5)	68 (35.4)	
Gravidity			< 0.01
Number Total = 293	99	194	
	1.25 ± 1.72	2.50 ± 1.61	
Parity			< 0.01
Number Total = 293	99	194	
	0.88 ± 1.30	2.03 ± 1.43	
Abortion			0.06
Number Total = 293	99	194	
	0.27 ± 0.69	0.43 ± 0.73	
Dysmenorrhea			
Number Total = 244	98	146	< 0.01*
Normal	36 (36.73)	47 (24.23)	
Severe	32 (32.65)	11 (5.67)	
Not	30 (30.61)	88 (45.36)	
Menes			0.97
Number Total = 244	98	146	
Regular	70 (71.43)	104 (53.61)	
Irregular	28 (28.57)	42 (21.65)	
Bleeding			0.41
Number Total = 244	98	146	
Normal	58 (59.18)	82 (42.27)	
Severe	29 (29.59)	53 (27.32)	
Low	11 (11.22)	11 (5.67)	

	Endometriosis N = 100	Normal N = 197	P-value
BMI, body mass index			
P-value < 0.05 is significant			

There was no significant difference in the mean age between the endometriosis patients and the controls group ($P = 0.9$) while cases had significant lower BMI compared to control group ($P < 0.01$).

Both groups were showed significant differences in gravidity and parity ($P < 0.01$). Conversely, no significantly different was observed in abortion between the two groups ($P = 0.06$).

Patients with endometriosis showed some symptom of endometriosis such as dysmenorrhea higher than the control group ($P < 0.01$). A significant difference was not found in the bleeding ($P = 0.41$) and menes between the two groups ($P = 0.97$).

TGF-ß1 variant (-509 C/T) in Patients with Endometriosis and Controls

Totally, 100 cases and 196 controls from 297 enrolled subjects were successfully genotyped for − 509 C/T variant in the promoter of *TGF-ß1* gene.

The genotype distribution of -509 C/T variant did not show any deviation from the HWE in control subjects ($P = 0.498$).

The genotype and allele frequencies for the *TGF-ß1* -509 C/T SNP are summarized in Table 3.

Table 3
Comparison of un-adjusted and adjusted odds ratio for five genetic models for candidate gene variant

Gene (rsID)	Genotype/allele	Case N = 100	Control N = 196	P-value	OR (95 % CI) TT vs. CC (Homozygote)		OR (95 % CI) CT vs. CC (Heterozygote)		OR (95 % CI) CT + TT vs. CC (Dominant)	
TGF-β1 (rs1800469)	CC	21 (21)	32 (16.33)	0.452	Un-Adjusted	Adjusted	Un-Adjusted	Adjusted	Un-Adjusted	Adjusted
	CT	44 (44)	100 (51.02)		0.833 (0.418–1.65) P = 0.603	0.303 [b] (0.117–0.781) P = 0.014	0.670 (0.348–1.29) P = 0.231	0.427 [a] (0.186–0.981) P = 0.045	0.734 (0.397–1.35) P = 0.322	0.366 [b] (0.168–0.796) P = 0.011
	TT	35 (35)	64 (32.65)		OR (95 % CI) T vs. C (Allelic)		OR (95 % CI) TT vs. CT + CC (Recessive)			
	C	86 (43)	164 (41.84)	0.786	Un-Adjusted	Adjusted	Un-Adjusted	Adjusted		
	T	114 (57)	228 (58.16)		0.953 (0.675–1.34) P = 0.786	0.545 [a] (0.352–0.844) P = 0.007	0.900 (0.541–1.469) P = 0.686	1.935 [b] (1.00-3.720) P = 0.048		

OR, odds ratio; CI, confidence interval

[a] Adjusted for Age, BMI, parity, abortion

[b] Adjusted for age, BMI, parity

A significant difference was not observed in the CT and TT genotype distributions of *TGF-ß1* -509 C/T between the cases and controls (P = 0.4). No significant result was also found between genotype frequency of these groups after merging heterozygous and homozygous mutant genotypes (CT + TT). The frequency of the CT, TT genotypes in the patients was significantly higher than the control group after adjusted for some quantitative variables (Adjusted CT: OR = 0.427, 95% CI = 0.186–0.981, P = 0.045; Adjusted TT: OR = 0.303, 95% CI = 0.117–0.781, P = 0.014).

The combined CT + TT genotype was associated with a significantly decreased risk of endometriosis compared with the CC genotype (Adjusted: OR = 0.366, 95% CI = 0.168–0.796, and P = 0.011).

There were no significant differences in the allele frequencies of *TGF-ß1* -509 C/T between the cases and controls ($P = 0.786$). The presence of T allele appeared to be a protective allele for the development of endometriosis after multivariate analysis for age, BMI, parity, and abortion using regression test (Adjusted: OR = 0.545, CI = 0.352–0.844, P = 0.007).

Analysis of mRNA TGF-ß1 Expression

As shown in Fig. 1a, no significant differences were observed between endometriosis patients and controls in TGF-ß1 mRNA expression ($P = 0.32$).

Evaluating the expression of TGF-ß1 level through the menstrual cycle also showed that in the proliferative phase, TGF-ß1 mRNA level in eutopic endometrium from women with endometriosis was not significantly different compared to the controls ($P = 0.10$) (Fig. 1b). In the secretory phase, endometrium tissue from women with the disease also showed similar mRNA expression compared to the controls ($P = 0.96$) (Fig. 1c). On the other hands, we found that endometrial tissue from controls showed higher TGF-ß1 mRNA levels in the proliferative phase than in the secretory phase but the results was not significant ($P = 0.20$) (Fig. 1d). Besides that, no significant difference was observed in TGF-ß1 level when comparing secretory versus proliferative phase in endometrium from women with endometriosis ($P = 0.18$) (Fig. 1e).

Discussion

In the present study, we undertook genotype analysis of the − 509 C/T variant and mRNA expression of the *TGF-β1* gene in relation to endometriosis and found that presence of *TGF-β1* 509T allele was protective against development of endometriosis (decreasing 46% risk of endometriosis), while patients with C allele were prone to have endometriosis after adjustment for cofounding variables.

According to the first investigation for the role of -509 C/T variant in susceptibility to endometriosis in a study by Hsieh and colleagues it has been demonstrated that TT genotype and T allele of *TGF-β1* gene are related to the higher risk of advanced endometriosis in the Taiwanese women. But, no association of this variant and late-stage endometriosis was reported in their study. Based on their results, *TGF-β1* may be a candidate gene for endometriosis risk. So that, genotype and allele frequencies of *TGF-β1* variant can be as convenient markers for the prediction of endometriosis susceptibility [10]. Further, the contraversial result has been identified by Van Kaam et al. They did not observe any relationship between this variant and advanced-stage endometriosis in Dutch women. They also studied the role of *TGF-β1* gene variant in increased risk of deep infiltrating endometriosis and no evidence of significant association was found [16].

Also, Kim and colleagues reported that the *TGF-β1*-509 C/T variant may not be associated with an increased risk for advanced-stage endometriosis in Korean population. They did not observe significant differences in the genotype distributions or allele frequencies of the *TGF-β1* gene − 509 C/T variant between endometriosis patients and controls [15].

Considering the findings of the study by Lee et al the TC haplotype allele of the − 509 C/T variants in the *TGF-β1* gene may be associated with an increased risk of early-stage endometriosis and is thus one genetic factor that may determine susceptibility for endometriosis [17].

These findings corresponded to the result of one Meta-analysis of six case-control studies that showed no positive correlation of -509 C/T variant with risk of endometriosis [9]. Similarly, no significant association was identified concerning the − 509 C/T variant with endometriosis development in a recent meta-analysis [18].

In disagreement with only previous report by Johnson and colleagues and in accordance with the statistical results, our data indicate that there were no significant differences in mRNA TGF-β1 between endometriosis patients and controls. Finding of Johnson study

demonstrates lower TGF-β1 mRNA and protein levels in endometrium with endometriosis than controls during the mid-secretory phase [19].

Conclusion

Taken together, our study findings provided some evidence that the *TGF-β1* gene variant shows a significant protection in endometriosis susceptibility in an Iranian population. Clinical data and adjustment for co-founding factors seems to be a very important issue and must be considered in the interpretation of results.

However, no association between TGF-β1 expressions in endometrial tissue and endometriosis risk was observed.

Since the lack of association might come from the small sample size, which is the main limitation in our study therefore further investigations with a larger sample size including larger groups of patients with the same stage of endometriosis should be performed so that peritoneal fluid (PF) and peripheral blood could be obtained during the same stage of the menstrual cycle.

Declarations

Funding

No funding was obtained.

Compliance with ethical standards

Conflict of interest

There was no conflict of interest to report.

Ethical approval

The study was approved by the ethics committee of Tehran University of medical sciences.

Informed consent

Informed consents were obtained from the study participants.

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Figures

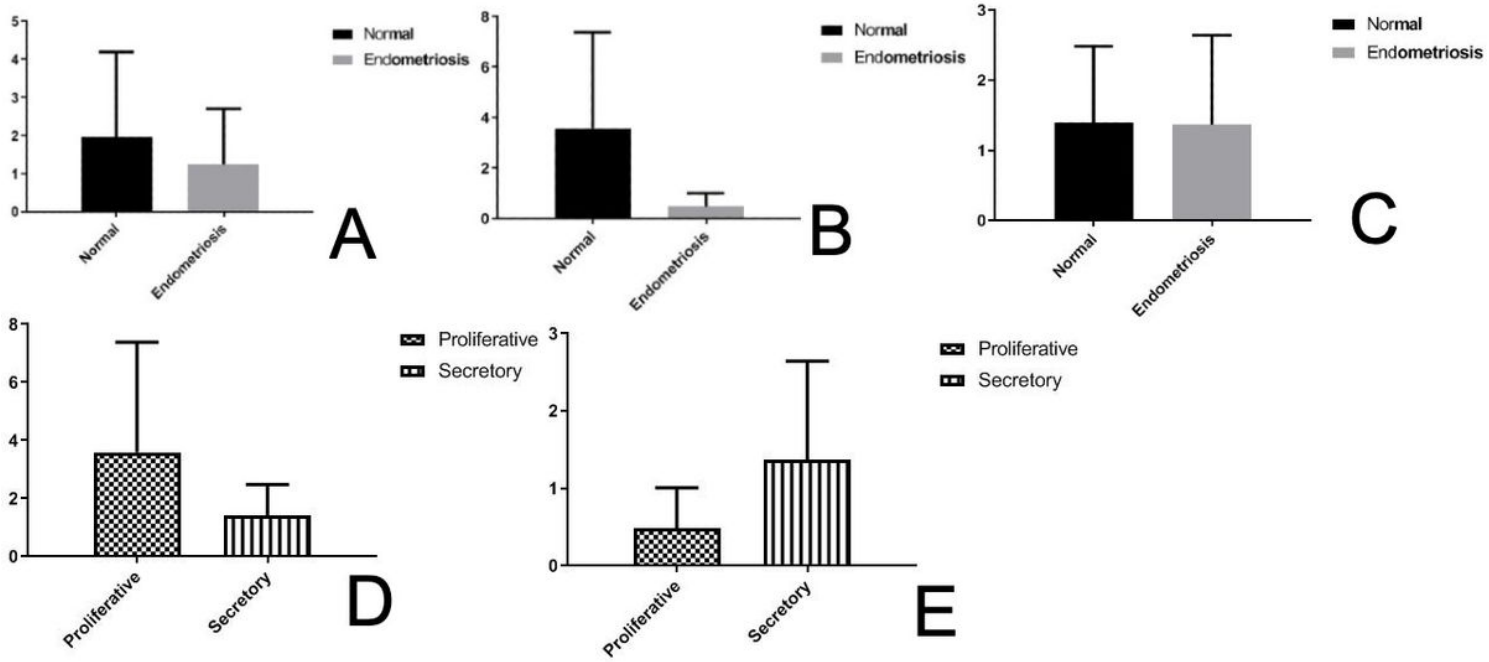

Figure 1

The mRNA expression differences of TGF-β1gene in eutopic endometrium tissue of endometriosis patients and normal subjects an also proliferative and secretory phases of the menstrual cycle. a) The mRNA expression differences between endometriosis and normal subjects, b) The mRNA expression differences between endometriosis and normal subjects in proliferative phase, c) The mRNA expression differences between endometriosis and normal subjects in secretory phase, d) The mRNA expression differences between proliferative and secretory phases in normal subjects, and e) The mRNA expression differences between proliferative and secretory phases in endometriosis patients.