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ORIGINAL ARTICLE

Association of two Single Nucleotide Polymorphisms (SNPs) of IL-16 gene with the Development and Grading of Endometriosis: A Case Control Study

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ABSTRACT

Background: *Endometriosis is an inflammatory disease depends on estrogen; The endometrium migrates outside the uterine cavity to different sites but most common to the pelvic cavity so it may cause pelvic pain and infertility. In recent years the effect of epigenetics in development of endometriosis has been studied widely. Interleukin16 (IL-16) is a cytokine that has multiple functions, it was originally functioned as a chemotactic factor. Several SNPs such as rs1131445, rs11556218, rs4778889 and rs4072111 are recognized in IL-16, and genetic studies have reported the probable association of these genetic variations with patients' liability to endometriosis.* ***Objectives:*** *We performed this study to question if the rs4072111 and rs11556218 SNPs of the IL-16 gene were related to development and grading of endometriosis in Egypt or not, also to use these SNPs, if they have a role, as predicting factors to measure the chance of getting endometriosis among women in the future.* ***Methodology:*** *This case control study consists of 189 women (94 patients and 95 controls), extraction and amplification of the genes of interest were performed, followed by analysis for the presence or absence of SNPs in IL-16 gene, which are rs4072111 (Pro434Ser) and rs11556218 (Asn446Lys) by RFLP, using the enzymes Bsmai and NdeI respectively.* ***Results:*** *A significant association was found between presence of G allele of rs11556218 with development and grading of endometriosis, on the other hand no significant association was found regarding the rs4072111 SNP with the development or grading of endometriosis.* ***Conclusion:*** *IL- 16 gene polymorphism is associated with the development of endometriosis and can be used as a predictor risk factor for susceptibility of endometriosis.*

INTRODUCTION

Endometriosis is an inflammatory disease depends on estrogen. The endometrium migrates outside the uterine cavity to different sites but most common to the pelvic cavity so it may cause pelvic pain and infertility[1]. It is reported to affect up to 6 to 10% of females in reproductive age [2], but symptoms vary widely between individuals [3], they may include continuous pelvic pain, uterine bleeding [3], also urinary tract and gastrointestinal symptoms may be present[4]. Endometriosis is a major problem of female's health; it has a great effect on the quality of life.

Multiple factors aid in the occurrence of this disease, this may include genetic and environmental ones. But, the specific molecular and pathophysiological pathways causing endometriosis are still not obvious [5]. Not all cases of endometriosis have the same theory. Some researchers found that genetic factors cause the heritability of endometriosis[6]. In recent years the effect of epigenetics in development of endometriosis has been studied widely and specific steps regarding DNA methylation and histone post-translational modifications have been detected [7]. The change of gene expression plays a significant role in the development of endometriosis. Recognition of different loci involved in the development of endometriosis has been done through candidate gene association researches, genome-wide association researches and different meta-analysis studies[8-10]. The number of new endometriosis-associated loci is increasing taking in consideration the importance of the severe stages of the disease (stage III/IV endometriosis) [8,11]. Previous studies found that different cytokines can be used for the diagnosis of endometriosis, because they were found in the serum and peritoneal fluid [12].

Because of the complicated aspects of the disorder, finding a safe noninvasive method to aid in diagnose of endometriosis is very important. Many researches measured the serum and peritoneal fluid levels of cytokines in the endometriosis patients, and some of

them reported possible biomarkers that may help in both diagnosis and prognosis of the condition [13].

Interleukin16 (IL-16) is a cytokine that has multiple functions, it was originally functioned as a chemotactic factor. IL-16 and different cytokines like IL-1, IL-6, IL-8, IL-11, IL-17, Eotaxin and Tumor necrosis factors (TNF) (α and β) are considered portion of chronic inflammation cytokines [14]. These cytokines function as enhancers of systemic or tissue specific inflammation. Change in synthesis of these cytokines can alter the tissue fixed cells like macrophages and endothelial cells, and cause a systemic response to an inflammatory reaction[15]. Not only IL-16 is known as lymphocyte chemoattractant factor but also it has been considered as a coordinator for the immune system machinery [16].

It is synthesized by different cell types, this cytokine plays an important role in the regulation of cellular functions. The specific technique of action of this cytokine as an inflammatory mediator is not really known. It is assumed that IL‑16 activates T lymphocytes, leading to secretion of different cytokines[17].

Several SNPs such as rs1131445, rs11556218, rs4778889 and rs4072111 are recognized in IL-16, and genetic studies have reported the probable association of these genetic variations with patients' liability to different diseases [18]. Two SNPs in this gene (rs4072111 C/T and rs11556218 T/G) were found to be related to various autoimmune diseases [19]], as well as with various types of cancer [20,21]. rs11556218 SNP produces an amino acid change (asn446lys) on location 446 of the shorter isomorph 2 (631 aminoacids) of Pro‑IL‑16, that may change protein structure and function. the rs4072111 is a different missense SNP (Pro434Ser) occurring on position 434 of the longer isomorph 1[22].

We performed this study to question if the rs4072111 and rs11556218 SNPs of the IL‑16 gene were related to development and grading of endometriosis in Egypt or not, also to use these SNPs, if they have a role, as predicting factors to measure the chance of getting endometriosis among women in the future.

METHODOLOGY

Study design and patient selection:

This case control study consisted of 189 women (94 patients and 95 controls), who visited outpatient Clinics in Obstetrics and Gynecology Department of Zagazig University Hospitals, Egypt, complaining from pelvic pain and/or infertility. Exclusion criteria included patients complaining from arthritis either, rheumatoid or giant cell, retinopathy caused by diabetes and psoriasis. The women in this study were subjected to diagnostic laparoscopy or laparotomy to either confirm or exclude the presence of endometriosis by histopathological examination of biopsies taken to determine the presence or absence of endometriosis and also to stage the degree of endometriosis according to American Fertility Society Classification [23]. The study was done in the Microbiology and Immunology Department Faculty of Medicine Zagazig University in corporation with Obstetrics and Gynecology Department Faculty of Medicine Zagazig University from May 2016 to September 2017.

Ethics status

This study was approved by the Institutional Review Board (IRB)- Faculty of medicine, Zagazig University. An informed written consent was obtained from all women at time of recruitment and their data was confidentially maintained.

Analysis of two SNPs of IL16 gene:

1- *Extraction amplification of genes of interest*

Genomic DNA was extracted from peripheral blood. Blood samples were collected into EDTA tubes, the extraction was done using the QIAamp Blood Kit (QIAGEN, Hilden, Germany), according to manufacturer instructions. The extracted DNA was stored until be used at ‑20˚C. Amplification of the segments carrying the polymorphic sites was carried out using polymerase chain reaction. The following primer pairs were used, the upstream primers 5'‑CAC TGT GAT CCC GGT CCA GTC‑3' and 5'‑GCT CAG GTT CAC AGA GTG TTT CCA TA‑3' as well as the downstream primers 5'‑TTC AGG TAC AAA CCC AGC CAG C‑3' and 5'‑TGT GAC AAT CAC AGC TTG CCT G‑3', to generate the following segments: rs4072111 (164 bp) (c>t) and rs11556218 (171 bp) (t>G) of the IL‑16 gene, respectively. PCR was performed in a Thermocycler (Biometra, Germany). Polymerase chain reactions (PCR) were carried out according to Michail et al. [22]. A hot start was used with initial heating at 94˚C for 5 min and then 30 cycles of denaturing (at 94˚C for 30 sec), annealing for 30 secs (60˚C for rs11556218 and 60˚C for rs4072111) and chain extension (at 72˚C for 30 sec), followed with a final extension step at 72˚C for 5 min.

Quality control measures for PCR

- Positive control: Internal Quality control kit was used (Investigator 24plex QS Kit) (Qiagen, Germany)
- Negative control: Dnase/Rnase free water was added to the master mix solution instead of target nucleic acid. To ensure that the master mix and the processing reagents are not contaminated.

2- *Restriction fragment polymorphism (RFLP):*

The selection of the two SNPs was done according to the previous studies which found an association of theses polymorphisms with the presence and severity of endometriosis[24]. The two SnPs in the IL‑16 gene, rs4072111 (Pro434Ser) and rs11556218 (Asn446Lys) were typed by restriction fragment length

polymorphism, the enzymes used were Bsmai and NdeI (Fermantas, UK). The reaction was performed in a tube containing 30 µl, of which 10 µl of PCR product, 1µl of restriction enzyme, 2 µl of fast digest buffer and complete the rest with 17 µl Dnase free water, then incubate the tube for 10 min at 37°C, analysis of the results was done according to [25].

Both undigested and digested PCR products were visualized in 2.5% agarose gel stained with ethidium bromide and 50bp DNA ladder. (Qiagen, Germany, GmbH).

Quality control for RFLP:

- Positive control: A pre-made restriction enzyme map obtained from nebcutter (New England biolabs) was used to confirm the performance of the restriction enzymes used.
- ***Negative control:***
  - A sample of the amplicon, prepared and handeled in a similar way as the positive control, but without adding the enzyme, this was done to confirm that digestion of the DNA segments resulted from the action of the restriction enzyme, and not contaminating enzymes.
  - Dnase/Rnase free water was added to the master mix in place of nucleic acid. To ensure that the master mix and final processing reagents are not contaminated.

Other methods to avoid contamination and disruption of PCR techniques were also applied: using laminar air-flow hoods in nearly all steps, use of bleach solution to disinfect the surfaces and use of filtered tips and perform different steps in different areas.

Statistical Analysis

Statistical analysis was done by SPSS version 25. Data was presented as frequency and proportion. Chi square test and odds ratio was used to analyze data. Receiver operating characteristic (ROC) curve was used to evaluate the performance of studied SNPs as biomarkers for determination of women's susceptibility for development of the disease. P values less than 0.05 were considered as significant.

RESULTS

In the present study it was found that only 8 patients with (8.5%) of cases were in stage I, also 11 patients were in stage II, and the majority of cases were of stages III and IV, with 34 (36.2%) patients in stage III, and 41 (43.6) patients in stage IV (table 1).

Table 1: Frequency of different endometriosis stages according to ASAI:

Stage	I	II	III	IV	Total
No. of patients	8 (8.5%)	11 (11.7%)	34 (36.2%)	41 (43.6%)	94

Regarding the IL-16 rs11556218 SNP analyzed, it was found that there was a statistically significant difference between both the G/G, G/T genotypes and G allele in patients than controls, while no statistically significant difference between T/T genotype or T allele between both patients and controls (table 2).

Table 2: Frequency of different genotypes and alleles of the IL-16 rs11556218 SNP analyzed in 189 women (94 patients and 95 controls):

rs11556218	Patients	Controls	p value	OR (95% CI)
Genotypes	n= 94	n= 95		
G/G	37 (39.4%)	19 (20.0%)	**< 0.001**	8.0 (3.7 – 17.7)
G/T	42 (44.7%)	14 (14.7%)	**< 0.001**	12.4 (5.4 – 28.4)
T/T	15 (15.9%)	62 (65.3%)		1.0 (Reference)
Alleles	n= 188	n= 190		
G	116 (61.7%)	52 (27.4%)	**< 0.001**	
T	72 (38.3%)	138 (72.6%)		1.0 (Reference)

In table (3), the relation between IL-16 rs11556218 SNP, and the stages of endometriosis was analyzed, it was found that also there is a statistically significant difference between both the G/G, G/T genotypes and G allele in patients with stages III, and VI, than controls, while no statistically significant difference between T/T genotype or T allele between both patients with stages III, and VI and controls.

Table 3: Frequency of different genotypes and alleles of the IL-16 rs11556218 SNP analyzed in 75 women with endometriosis (stage III and IV) and 95 healthy controls.

rs11556218	Patients	Controls	p value	OR (95% CI)
Genotypes	n= 75	n= 95		
G/G	32 (42.7%)	19 (20.0%)	**< 0.001**	13.1 (5.2 – 33.1)
G/T	35 (46.7%)	14 (14.7%)	**< 0.001**	19.4 (7.4 – 50.7)
T/T	8 (10.6%)	62 (65.3%)		1.0 (Reference)
Alleles	n= 150	n= 190		
G	99 (66.0%)	52 (27.4%)	**< 0.001**	5.2 (3.2 – 8.2)
T	51 (34.0%)	138 (72.6%)		1.0 (Reference)

In the current research, IL-16 rs4072111 SNP studied showed no statistically significant difference between patients and controls regarding any genotype or allele (table 4).

Table 4: Frequency of different genotypes and alleles of the IL-16 rs4072111 SnP analyzed in 189 women (94 patients and 95 controls):

rs4072111	Patients	Controls	p value	OR (95% CI)
Genotypes	n= 94	n= 95		
C/C	73 (77.7%)	69 (72.6%)	0.3	1.9 (0.5 – 6.6)
C/T	17 (18.1%)	19 (20.0%)	0.5	1.6 (0.4 – 6.3)
T/T	4 (4.2%)	7 (7.4%)		1.0 (Reference)
Alleles	n= 188	n= 190		
C	163 (86.7%)	157 (82.6%)	0.2	1.4 (0.8 – 2.4)
T	25 (13.3%)	33 (17.4%)		1.0 (Reference)

Also, it was found that IL-16 rs4072111 SNP wasn't associated at all with the stage of endometriosis, not only at the genotype level but also at the allele level (table 5).

Table 5: Frequency of different genotypes and alleles of the IL-16 rs4072111 SNP analyzed in 75 women with endometriosis (stage III and IV) and 95 healthy controls:

rs4072111	Patients	Controls	p value	OR (95% CI)
Genotypes	n= 75	n= 95		
C/C	60 (80.0%)	69 (72.6%)	0.06	6.1 (0.7 – 50.9)
C/T	14 (18.7%)	19 (20.0%)	0.1	5.2 (0.6 – 46.8)
T/T	1 (1.3%)	7 (7.4%)		1.0 (Reference)
Alleles	n= 150	n= 190		
C	122 (81.3%)	157 (82.6%)	0.8	0.9 (0.5 – 1.6)
T	28 (18.7%)	33 (17.4%)		1.0 (Reference)

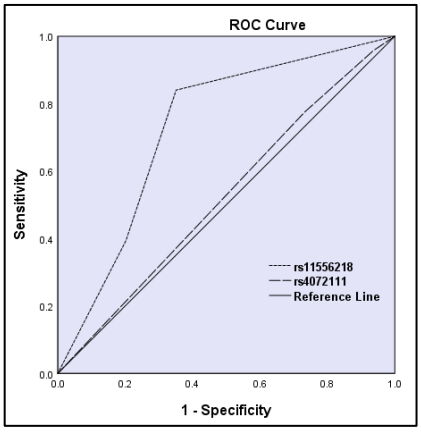

Fig. 1: ROC curves for rs11556218 SNP and rs4072111 SNP risk.

Diagnostic performance of IL 16 SNPs

SNP	AUC	95% CI	P
rs11556218	0.73	0.62 – 0.80	**< 0.001**
rs4072111	0.52	0.44 – 0.61	0.6

We performed receiver operating characteristic (ROC) curve was used to evaluate the performance of studied SNPs as biomarkers for determination of women's susceptibility for development of the disease, and it was found that IL-16 rs11556218 SNP has more discriminating power than IL-16 rs4072111 SNP (fig.1).

DISCUSSION

The specific mechanisms by which endometriosis occur are still not fully clear. Many factors precipitate to the development of endometriosis including patient lifestyle, environmental factors and genetic constitution which is considered the most important [26]. Stilley and his colleagues [27] found that individual variations in genetic determinants such as SNPs can alter patient's susceptibility to endometriosis. Nowadays the great advances in the study of human genetics and epigenetics researches revealed different genetic risk factors associated with the development of endometriosis. It was found that IL-16 is involved in a way or another in the pathogenesis of some immune disorders like tumors and some infections[28]. In the current research we questioned the impact of two SNPs (rs4072111 and rs11556218) of the IL‑16 gene and their relation with the development and grading of endometriosis, also the possible role of polymorphisms as susceptibility markers for endometriosis. In spite of detection of several polymorphisms in gene loci that were linked to development of endometriosis, it is obvious that there is great variability in theses loci polymorphisms according to different populations, so, it is a must to study these genetic polymorphisms in different populations [29]. In this study, we found that rs11556218 SNP alone is considered as a risk factor for the development and grading of endomeriosis. In Iran a study was performed about this genetic polymorphism and it was found that genotype and allelic distribution in the two IL‑16 SNPs rs4072111 and rs11556218 was significantly different between endometriosis patients and controls [30]. On the other hand, these polymorphisms didn't appear to affect the development of endometriosis in a Chinese [31] or a Korean population [32]. These findings show the benefit of studying genetic variation in different populations in a trial to rule out or accuse genetic basis of endometriosis. As known, change in DNA bases of IL-16 may results in change in cytokine production or effect, and this change may alter susceptibility to endometriosis [22]. Many researches were conducted and revealed that IL-16 exhibited elevated levels in patients than control [33], but few of them questioned the mechanisms involved in this association [34,35]. However, in another study, the levels of IL‑16 in peritoneal fluid and sera of patients were less than those of the healthy controls' although the difference wasn't statistically significant [36]. Apparently, more studies are needed to identify the importance IL16 in association with endometriosis, and these studies should be performed on different populations. Functional mechanisms could be studied by gene expression and epigenetic analyses, in addition to genetic polymorphisms should be examined, in a trial to understand the real pathway of endometriosis development.

Association of endometriosis with epigenetic studies must be linked with the severity of the disease itself. In the present study we tried to compare the polymorphism in these two positions with the stages of the disease according to ASAI classification that divided the stages of the disease into 4 groups, we tried to question the association of the of these two SNPs with stage I and II as a group and stage II and VI as a separate group. In this current research we found that the G allele of IL-16 rs11556218 SNP is considered the risk factor for development of the sever grades of endometriosis. This finding was in accordance with [22], also they found that the SNPs of IL‑16 rs11556218 was considered a risk factor for severity of endometriosis. While there was no association between IL-16 rs4072111 SNP and the severity or the development of endometriosis, this also was in accordance with [22]. On the other hand, it was contrary to the report of Azimzadeh et al. [24, as they found that IL-16 rs4072111 SNP was associated with the development and grading of endometriosis. By analysis of ROC curve, in an attempt to predict the women with greater chance to develop endometriosis, we found that IL-16 rs11556218 SNP has more discriminating power than IL-16 rs4072111 SNP, this was in agreement with Azimzadeh et al [24].

CONCLUSION & RECOMMENDATIONS

A significant association was found between presence of G allele of rs11556218 with development and grading of endometriosis, on the other hand no significant association was found regarding the rs4072111 SNP with the development or grading of endometriosis, so IL-16 gene polymorphism is associated with the development of endometriosis and can be used as a predictor risk factor for susceptibility of endometriosis, so changes in the IL-16 gene sequence can alter women's susceptibility to endometriosis, also changes in its sequence can affect the grades of the disease itself, which enhance the progress of the disease to the more severe form.

Further studies are needed to confirm, not only the association of polymorphisms as risk factors for endometriosis but also its functional mechanisms. Researches in different races and different populations are required.

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