A Search to Identify Genetic Risk Factors for Endometriosis

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Problem
To search for molecular markers of endometriosis the following polymorphisms: p53 codon 72 Pro (apoptosis), TNF alpha-308 (inflammation), VEGF-1164AA (angiogenesis), and SOD2 (oxidative stress) were investigated.

Method of study
Forty-two women—24 with surgically proven endometriosis and 18 with no endometriosis found at the time of laparoscopy–had buccal swabs taken for DNA analyses of 4 gene polymorphisms including p53codon72, TNF-308 G/A, VEGF-1154G/A, SOD Ala16Val DNA. The frequencies of genotypes and alleles of these polymorphisms were compared between women with and without endometriosis.

Results
No specific gene mutation differences for the four genes tested nor differences in the frequencies of heterozygous and homozygous mutations were found between patients with endometriosis and controls. In addition, no differences in allelic frequencies of the four genetic polymorphisms were observed between patients with endometriosis and control.

Conclusion
Endometriosis is not associated with gene mutations for p53codon72, TNF-308 G/A, VEGF-1154G/A, SOD Ala16Val.

Introduction

Endometriosis is a common gynecologic disease affecting around 10% of women in their reproductive years.[1] Among infertile women, its prevalence increases to 30% to 40%.[2] Currently, the diagnosis of endometriosis is based on the histologic identification of endometrial glands and stroma using invasive procedures such as laparoscopic surgery.[2] Several non-invasive tests for endometriosis have been proposed,[3] but none have proved clinically useful. Thus, a non-invasive tool for diagnosis of endometriosis is urgently needed.

Endometriosis has long been recognized as showing heritable tendencies, with a 5- to 7-fold greater incidence risk for first-degree relatives.[4] Several reports had indicated that women with a family history of endometriosis tend to have more advanced stages of endometriosis, associated with severe pelvic pain, and other related symptoms.[5] A search for candidate genes on which to base an endometriosis-specific test has been challenging. Mechanisms involved in the clinical manifestations of the disease have included problems with apoptosis,[6] immune responses,[7] and oxidative stress.[8] Using published results obtained by microarray technology[7] and

knowledge of the mechanisms involved in the disease,[9] we investigated the following genes in the hopes of identifying molecular markers for endometriosis: p53codon72, TNF-308 G/A, VEGF-1154G/A, SOD Ala16Val DNA.

Materials and methods

Patients

Forty-two women undergoing laparoscopy signed consent forms to participate in the study. Of the 42 women, 24 had histologic proven endometriosis confirmed by pathology and 18 had no endometriosis found at the time of laparoscopy. Among the 24 women with tissue diagnosis of endometriosis, 3 were classified as Stage 1, 2 Stage 2, 2 Stage 3, and 17 Stage 4. All women had their cheeks swabbed with a cotton swab to collect cells for DNA analyses of four gene polymorphisms including p53codon72, TNF-308 G/A, VEGF-1154G/A, SOD Ala16Val DNA. Institutional Review Board and informed consent were obtained from all participants.

DNA Extraction and PCR Analysis

Deoxyribonucleic acid (DNA) was isolated from buccal swabs collected from each patient, using the Promega DNA IQ Casework Pro Kit for Maxwell 16. DNA was analyzed using polymerase chain reaction (PCR). PCR amplification of the DNA, Taq Polymerase, deoxynucleotide mix, and standard reaction buffer were obtained from New England BioLabs, and thermal cycling was performed in a GeneMate Genius (Techne) thermal cycler. Thermal cycling parameters were 95.0°C/2 min; 37 cycles of:95.0°C/1 min, 53.5°C/30 s, 72.0°C/1 min; 72°C/5 min; 4.0°C. The oligodeoxynucleotide primers used for each marker were as follows (F = Forward primer; R = Reverse primer). Primers were purchased from Eurofins MWG Operon: p53 codon 72 (Pro/Arg; Arg = GCG, Pro = GGG), NCBI Reference Sequence: NT_010718.16, (F) 5′-GTAGCTGCCCTGGTAGGT-3′; (R) 5′-TCCCAAGCAATGGATGATTTGA-3′; TNF-308 (G>A) DNA sequence reference (16); (F) 5′-TCAGGACTCAACACAGCTT-3′, (R) 5′-TGTCTCGGTTTCTTCTCCAT-3′;VEGF-1154 (G>A), NCBI Reference Sequence: NG_008732.1 Location of variant (−1154) (16) (F) 5′-CACGTAACCTCACTTTCCTGCTC-3′, (R) 5′-TCCCCGCTACCAGCCGACT-3′; SOD Ala16Val (2246C>T), NCBI Reference Sequence: NG_008729.1, (F) 5′-AGGGAGGGGACGCGGGGACT-3′, (R) 5′-ACGTTCAGGTTGTTCACGTA-3′.

Polymerase chain reaction products were purified using the Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and sent to the Northwestern University Genomics Core Facility for DNA sequencing. This was accomplished using an ABI 3730 sequencer and BigDye Terminator v3.1 cycle sequencing kit. Results were analyzed using Applied Biosystems Sequence Scanner software v1.0.

Statistical Analysis

The frequencies of genotypes and alleles of p53 condon 72 Pro, TNF alpha-308, VEGF-1164AA, and SOD2 polymorphisms were compared between women with and without endometriosis using 2 × 2 contingency tables with Fisher's exact test (GraphPad InStat, San Diego, CA, USA). A two-tailed P value of < 0.05 was considered significant.

Results

Data from patients with endometriosis were compared with individuals without endometriosis (Table I). No significant differences were noted in age between the two groups. Women with endometriosis had lower gravidity than controls although this did not reach significance ($P = 0.07$). The frequency of infertility was higher among women with endometriosis compared with controls. Twelve sets of two-by-two contingency tables were set up for each marker comparing homozygous, heterozygous, and total number of mutations as well as alleles among patients and controls. The results of these analyses are shown in Figs 1 and 2.

The frequencies of heterozygous mutations among 4 genes tested are shown in Fig. 1a. No differences

Table I Clinical Characteristics of Women with and without Laparoscopically Proven Endometriosis

	Endometriosis	No Endometriosis	*P*
Age (years)			
Mean ± S.E.	33.8 ± 1.0	35.4 ± 1.8	NS
Range	24–43	25–49	NS
Gravidity			
Mean ± S.E.	1.3 ± 0.3	2.6 ± 0.7	0.07
Range	0–6	0–10	NS
Infertility (%)	73	28%	0.03

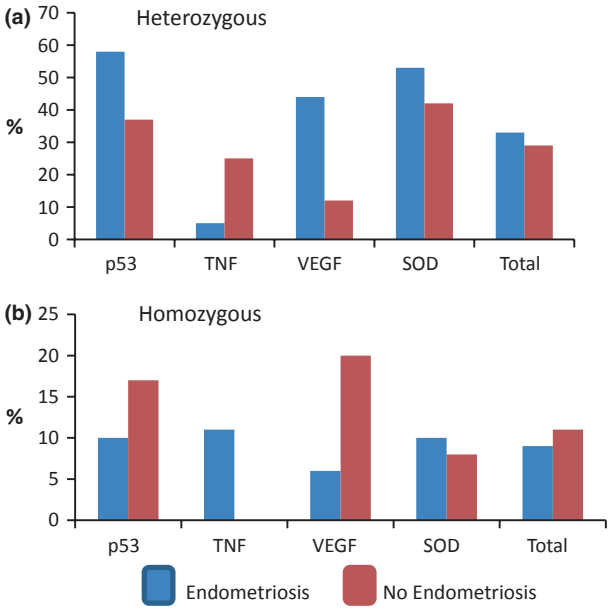

Fig. 1 Frequency of heterozygous (Panel a) and homozygous (Panel b) mutations for p53codon72, TNF -308 G/A, VEGF -1154G/A, SOD Ala16Val DNA among women with a diagnosis of endometriosis diagnosed by laparoscopy (blue bar) and women with no endometriosis found at laparoscopy (red bar). No significant differences between the two groups exist ($P > 0.05$).

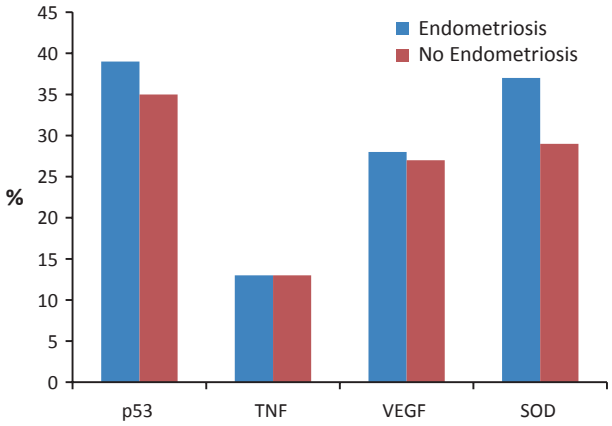

Fig. 2 Allelic frequency of mutations for p53codon72, TNF -308 G/A, VEGF -1154G/A, SOD Ala16Val DNA among women with a diagnosis of endometriosis diagnosed by laparoscopy (blue bar) and women with no endometriosis found at laparoscopy (red bar). No significant differences between the two groups exist ($P > 0.05$).

in specific gene mutations were observed when patients with endometriosis were compared with women without endometriosis. Comparison of the frequencies of homozygous mutations between women with endometriosis and without endometriosis (Fig. 1b) revealed no significant differences. When the total number of mutations was compared counting a heterozygous mutation as one gene mutation and a homozygous mutation as two mutations, again no differences were observed. Fig. 2 illustrates the allelic frequency of the 4 genetic polymorphisms. No differences in specific alleles were observed when patients with endometriosis were compared with women without endometriosis.

Discussion

The current study was undertaken to see whether genetic markers could identify individuals at risk of endometriosis. The rationale for the study was based on the perceived need for a non-invasive tool to diagnosis endometriosis and the observations that endometriosis showed heritable tendencies.[4] Furthermore, previous publications have reported problems with apoptosis,[6] immune responses,[7] and oxidative stress[8] to be involved in the mechanisms leading to the clinical manifestations of the disease. P53codon72 gene polymorphisms have been associated with defects in apoptosis. The p53 codon 72APro/Pro mutation interacts with p73 protein and inactivates its apoptotic function.[10] TNFα is a potent immunomediator and proinflammatory cytokine that has been implicated in the pathogenesis of a large number of human diseases including endometriosis.[8] A single nucleotide substitution of the TNFα gene at nucleotide-308 upstream from the transcription initiation site in the TNFα promoter is associated with elevated TNFα serum concentrations.[11] VEGF has been shown to induce inflammation as well as angiogenesis and is elevated in eutopic endometrium during the secretory phase in women with endometriosis compared with controls.[12] SODs are enzymes that provide protection against oxidative stress. SOD2 has manganese in its reactive center and is located in the mitochondria where it detoxifies superoxide radicals (O_2^-), a byproduct of oxidative phosphorylation, into hydrogen peroxide and O_2.[13] A common polymorphism associated with greater susceptibilities to pathologies is found in the mitochondrial leader targeting sequence (Val16Ala).[14]

When seeking the above genetic markers for endometriosis involved in apoptosis, inflammation, and angiogenesis, none were found. These findings were unexpected because apoptosis, inflammation, and angiogenesis are all processes that have been reported to be altered in favor of survival and

replenishment of endometriotic tissue.[9] Furthermore, endometriosis has been shown to be heritable with incidence in relatives of affected individuals up to seven times the incidence in women without such a family history.[4] While a few studies have reported single nuclear polymorphisms associated with endometriosis, others have been unable to find such associations.[15] Thus, identification of candidate genes related to endometriosis has been controversial and/or not realized as in the current study. These observations lead to the question of how can endometriosis display genetic transmission with no identification of genetic markers? Two explanations are obvious: Genes involved have not as yet been studied and epigenetic process is involved.

Epigenetic alterations have been shown to lead to many of the manifestations of endometriosis.[9] DNA methylation profiling has identified distinct targets that define some of the differences between eutopic and ectopic endometrium.[16] As a result of epigenetic hypomethylation and consequent overexpression of the nuclear steroidogenic factor-1 (SF1) and estrogen receptor β (ERβ), ectopic endometrium produces excessive estrogen and prostaglandins compared with eutopic endometrium.[16] The absence of expression of SF 1 receptor in the endometrium is determined in part by a classic cytosine-phosphate-guanine (Cpg) island at the promoter of SF 1 gene that is heavily methylated in endometrial stromal cells and unmethylated in endometriotic stromal cells.[16] Hypomethylation of a Cpg island at the promoter region of the estrogen receptor β gene causes high expression of the gene in endometriotic stromal cells, and hypermethylation silences it in endometrial stromal cells.[17] The high levels of ER β in endometriotic cells in turn lead to increased ER β binding to the progesterone receptor promoter and mediate its downregulation of expression of progesterone receptors.[18]

In conclusion, available data suggest that the mechanisms of cell survival employed by endometriotic cells including inflammation, angiogenesis, and apoptosis have been linked epigenetically rather than genetically to a stromal defect involving the excess formation of estrogen and prostaglandins as well as to progesterone resistance. As a result, no gene defects or polymorphisms have been confirmed.

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