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Analysis of inflammatory biomarkers IL-6, vascular endothelial growth factor and matrix metalloproteinases-9 expression in endometriosis

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Abstract

Objective: Special attention has been paid to genetic mechanisms that might have a significant impact on the context of the risk of developing endometriosis, in recent years. The study aimed to analyze the expression levels of three inflammatory biomarkers Interleukin-6 (IL-6), vascular endothelial growth factor, and matrix metalloproteinases-9, in the increased incidence of endometriosis.

Methods: The material for genetic testing was tissue slices embedded in paraffin blocks from these patients with endometriosis (I–II) ($n = 24$), endometriosis (III–IV) ($n = 24$), and the control group ($n = 30$) in Lianyungang maternal and child health hospital from January 2020 to December 2023. The expression levels of IL-6, vascular endothelial growth factor, and matrix metalloproteinases-9 genes were determined by the real-time polymerase chain reaction technique.

Results: The expression levels of IL-6 and vascular endothelial growth factor gene in the peripheral blood and peritoneal fluid of these endometriosis patients were not statistically significant lower than in the control group. Besides, the significant differences were found in IL-6, vascular endothelial growth factor and matrix metalloproteinases-9 between eutopic endometrial tissues of the endometriosis group, compared to the control group; and these increased significantly with the severity of the disease. In addition, there was significant difference in the expression level of matrix metalloproteinases-9 in peripheral blood and peritoneal fluid, and the difference was statistically significant in these patients with stages III–IV, compared with these patients with stages I–II. Among them, the Revised American Society for Reproductive Medicine classification of endometriosis was used in the group of patients with endometriosis.

Conclusion: These patients with endometriosis showed the significant differences in matrix metalloproteinases-9 expression in peripheral blood, peritoneal fluid, and eutopic and ectopic endometrial tissues as the condition worsens. The research suggested that the determination of matrix metalloproteinases-9 in peripheral blood has certain value in evaluating the condition of endometriosis, which might play an important role in the pathogenesis of endometriosis and be explored for postoperative recurrence monitoring.

Keywords

Inflammatory biomarkers, endometriosis, IL-6, VEGF, MMP-9

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Introduction

As an estrogen-dependent, gynecological disease, endometriosis is a very important medical and social problem because of the accompanying ailments and chronic nature.[1,2] Endometriosis is defined as an inflammatory process characterized during surgery by the presence of an epithelium and/or endometrial-like stroma outside the endometrium and

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myometrium, usually accompanied by an inflammatory process.[3] Endometriosis have complex clinical symptoms, mostly occurring in pelvic organs, mainly manifested as menstrual abnormalities, abdominal pain, infertility, and sexual intercourse pain.[4,5] Because these clinical symptoms are complex, it is difficult to make early clinical diagnosis, and its diagnosis is delayed.[6] The incidence rate in women of childbearing age is about 10%–15%.[2,7] Ovarian endometriosis (OEMs) is the most common of endometriosis, and its incidence rate is even as high as 50% in patients with infertility and dysmenorrhea.[8] Meanwhile, the recurrence after surgery has always been a focus of clinical attention, and repeated surgeries leading to decreased ovarian function directly affect women's fertility.[9,10]

The etiology of endometriosis is very complex, including hormonal, immunological, and environmental factors, as well as angiogenic and inflammatory factors, which jointly participate in the occurrence and development of endometriosis.[5,11] Among them, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP-2, MMP-9) play a crucial role in the destruction and degradation of extracellular matrix and angiogenesis.[12,13] The active proliferation of endothelial cells in ectopic endometrium and the significant increase in cytokine levels, including VEGF, IL-2, IL-6, IL-8, TNF-α, might promote the occurrence and development of endometriosis.[14,15] This study measured the concentrations of the inflammatory biomarkers VEGF, IL-6, and MMP-9 in the peripheral blood, peritoneal fluid, eutopic endometrium, and ectopic endometrium of patients with endometriosis at different stages compared to controls, to possibly correlate these inflammatory biomarkers with the risk of endometriosis. Thus, it aimed to find the effective biochemical measurement to help early detection of endometriosis and explore the prediction of disease recurrence.

Methods

Patients

In this prospective study, the study group consisted of 48 patients diagnosed with endometriosis by the laparoscopic surgery and pathological examination in Lianyungang maternal and child health hospital. On the other hand, the control group consisted of 30 patients who had no endometriosis during the surgical procedure, and the histopathological examination was confirmed. The exclusion criterion was concomitant cancer in patients preclassified to the study group and existing cancers in patients from the control group. Patients were selected for studies in the period between January 2020 and December 2023. The material for analysis was RNA isolated from paraffin blocks obtained from material collected during surgery or curettage of the uterine cavity in the department of gynecology, Lianyungang maternal and child health hospital. Paraffin blocks came from the archives of the department of clinical pathology of our hospital, and all preparations have been characterized histologically. The experiment complied with the Ethics Committee of Lianyungang maternal and child health hospital (LYG-ME202005), and a formal consent was obtained from the Bioethical Committee of Lianyungang maternal and child health hospital.

RNA isolation

According to the manufacturer's protocol, the total RNA was isolated and extracted from frozen formalin-fixed paraffin-embedded tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reverse transcribed to high-quality cDNA using a PrimeScript RT Master Mix (Vazyme Biotech, Nanjing, China). The specific methods are as follows: The tissue samples were placed in 2 mL Eppendorf tubes, dewaxed with 100% xylene, washed in 100% ethanol, and dried at 55℃ for 10 min. The dried tissue was suspended in 100 μL of paraffin tissue lysis buffer (included in the kit) and digested with proteinase K at 55℃ overnight. The resulting total RNA was used for cDNA synthesis or stored at −80℃ until use.

Real-time polymerase chain reaction

Total RNA was extracted from tissues using TRIzol (Invitrogen). Next, RNA was reverse-transcribed into cDNA, and polymerase chain reaction (PCR) was performed using SYBR Green PCR Kit (Takara; Otsu, Japan). All the experiments were performed in triplicate, and β-action was selected as the reference gene for mRNA. Relative gene expression levels were calculated using the 2 −ΔΔCT method. The list of primers for RT-qPCR is presented below:

IL-6

forward: 5′-ACTCACCTCTTCAGAACGAATTG- 3′,

reverse: 5′- CCATCTTTGGAAGGTTCAGGTTG- 3′;

VEGF

forward: 5′-AGGGCAGAATCATCACGAAGT- 3′,

reverse: 5′-AGGGTCTCGATTGGATGGCA- 3′;

MMP-9

forward: 5′-GGGACGCAGACATCGTCATC- 3′,

reverse: 5′-TCGTCATCGTCGAAATGGGC- 3′;

β-action

forward, 5′-CCGTAAAGACCTCTATGCC- 3′,

reverse, 5′-CTCAGT AACAGTCCGCCTA- 3′.

Table 1. Clinical characteristics of the patients included in this study.

Parameter	OEMs (I–II)	OEMs (III–IV)	Control	*p*-Value
Number of patients	24	24	30	
Age, year	31.29 ± 5.34	32.60 ± 5.16	35.10 ± 5.03	0.077
BMI, kg/m^2	22.36 ± 2.21	21.59 ± 2.02	22.73 ± 2.99	0.075
Gravidity, *n*	1.62 ± 0.91	1.41 ± 1.08	1.45 ± 0.92	0.569
Parity, *n*	1.07 ± 0.78	1.05 ± 0.73	0.86 ± 0.71	0.312
Menstrual frequency, *d*	28.57 ± 2.27	28.86 ± 1.86	29.50 ± 1.95	0.101
Menstrual time, *d*	5.52 ± 0.89	5.08 ± 1.08	4.31 ± 1.16	0.137

rASRM: the revised American society for reproductive medicine staging system; OEMs: ovarian endometriosis; BMI: body mass index.

Table 2. The expression of IL-6 in peripheral blood, peritoneal fluid, eutopic endometrium, and ectopic endometrium of case and control.

Parameter	OEMs (I–II)	OEMs (III–IV)	Control	*p*-Value
Number of patients	24	24	30	
Peripheral blood	226.73 ± 41.18	198.18 ± 17.75	234.94 ± 27.89	0.086
Peritoneal fluid	185.05 ± 29.47	202.60 ± 32.62	143.36 ± 14.14	0.216
Eutopic endometrium	0.5052 ± 0.02681	0.9154 ± 0.02183	1 ± 0.001962	**0.042**
Ectopic endometrium	9.618 ± 0.1395	11.782 ± 0.2314	—	0.153

The bold represents statistical differences.
rASRM: the revised American society for reproductive medicine staging system; OEMs: ovarian endometriosis.

Statistical analysis

Data are expressed as means ± standard deviations. Statistical analysis of the obtained results was carried out using the SPSS 22.0 (IBM, SPSS, Chicago, IL, USA). The analysis of the significant differences in the level of gene expression at the mRNA level was carried out using non-parametric tests (Mann–Whitney *U* test, Kruskal–Wallis test) due to the lack of normality of the distribution of the obtained results, which were confirmed by the Shapiro–Wilk test. The Chi-square (χ^2) test was used to analyze the correlations between gene expression and clinicopathological features. The student's *t*-test was used to determine differences between two groups. *p*-value less than 0.05 was considered to be statistically different.

Results

The clinical stage of the patients with endometriosis was determined according to the rASRM (The Revised American Society for Reproductive Medicine classification of endometriosis).[14,15] According to the rASRM, all patients were divided into two groups: endometriosis I–II groups and endometriosis III–IV groups, of which 24 cases were in the endometriosis I–II groups and 24 cases were in the endometriosis III–IV groups. The characteristics of these patients were shown in Table 1.

Analysis of inflammatory biomarker IL-6 expression in endometriosis

The expression of IL-6 in peripheral blood, peritoneal fluid, eutopic endometrium, and ectopic endometrium of case and control were summarized in Table 2. The peripheral blood of endometriosis I–II, endometriosis III–IV, and control group were (mean 226.73 ± 41.18), (mean 198.18 ± 17.75), and (mean 234.94 ± 27.89), respectively. And the peritoneal fluid of endometriosis I–II, endometriosis III–IV, and control group were (mean 185.05 ± 29.47), (mean 202.60 ± 32.62), and (mean 143.36 ± 14.14), respectively. Therefore, the expression level of the IL-6 gene from the peripheral blood and peritoneal fluid in endometriosis patients was not statistically significantly lower than in the control group. In addition, the significant differences were found in IL-6 between eutopic endometrial tissues. Among them, the eutopic endometrium of endometriosis I–II, endometriosis III–IV, and control group were (mean 0.5052 ± 0.02681), (mean 0.9154 ± 0.02183), and (mean 1 ± 0.001962), respectively (Eutopic endometrium: *p*-value = 0.042).

Analysis of inflammatory biomarker VEGF expression in endometriosis

Table 3 was showed the expression level of VEGF in peripheral blood, peritoneal fluid, eutopic endometrium, and

Table 3. The expression of VEGF in peripheral blood, peritoneal fluid, eutopic endometrium, and ectopic endometrium of case and control.

Parameter	OEMs (I–II)	OEMs (III–IV)	Control	*p*-Value
Number of patients	24	24	30	
Peripheral blood	257.6 ± 24.6	326.6 ± 7.8	158.9 ± 16.1	0.641
Peritoneal fluid	89.9 ± 6.9	122.7 ± 9.1	75.2 ± 6.2	0.332
Eutopic endometrium	0.5535 ± 0.003389	0.5136 ± 0.003289	1 ± 0.003314	**0.042**
Ectopic endometrium	3.228 ± 0.0355	6.828 ± 0.0435	—	**0.041**

The bold represents statistical differences.
rASRM: the revised American society for reproductive medicine staging system; OEMs: ovarian endometriosis.

Table 4. The expression of MMP-9 in peripheral blood, peritoneal fluid, eutopic endometrium, and ectopic endometrium of case and control.

Parameter	OEMs (I–II)	OEMs (III–IV)	Control	*p*-Value
Number of patients	24	24	30	
Peripheral blood	1090.22 ± 108	2430 ± 219.2	258.25 ± 49.3	<**0.0001**
Peritoneal fluid	1431.3 ± 96.6	2775.5 ± 112.2	589.8 ± 37.6	<**0.0001**
Eutopic endometrium	1.669 ± 0.05226	1.587 ± 0.05226	1.000 ± 0.03181	**0.0002**
Ectopic endometrium	2.664 ± 0.01993	2.984 ± 0.01993	-	0.315

The bold represents statistical differences.
rASRM: the revised American society for reproductive medicine staging system; OEMs: ovarian endometriosis.

ectopic endometrium of case and control. There were no statistically significant differences in the expression of VEGF gene between the endometriosis group and the control group. The peripheral blood and peritoneal fluid of endometriosis I–II, endometriosis III–IV, and control group were (mean 257.6 ± 24.6) and (mean 89.9 ± 6.9), (mean 326.6 ± 7.8) and (mean 122.7 ± 9.1), (mean 158.9 ± 16.1), and (mean 75.2 ± 6.2), respectively. Besides, there was significantly differences in VEGF expression level between eutopic endometrial tissues. The eutopic endometrium of endometriosis I–II, endometriosis III–IV, and control group were (mean 0.5535 ± 0.003389), (mean 0.5136 ± 0.003289), and (mean 1 ± 0.003314), respectively (p-value = 0.042), and, the ectopic endometrium of endometriosis I–II and endometriosis III–IV groups were (mean 3.228 ± 0.0355) and (mean 6.828 ± 0.0435), respectively (p-value = 0.041).

Analysis of inflammatory biomarker MMP-9 expression in endometriosis

The expression of MMP-9 in peripheral blood, peritoneal fluid, eutopic endometrium, and ectopic endometrium of case and control were summarized in Table 4. Statistically, there was a significantly higher expression of MMP-9 of the peripheral blood, and peritoneal fluid in cases of endometriosis patients compared to control group. Besides, the difference was statistically significant in these patients with stages III–IV, compared with these patients with stages I–II. The peripheral blood and peritoneal fluid of endometriosis I–II, endometriosis III–IV, and control group were (mean 1090.22 ± 108) and (mean 1431.3 ± 96.6), (mean 2430 ± 219.2) and (mean 2775.5 ± 112.2), (mean 258.25 ± 49.3), and (mean 589.8 ± 37.6), respectively (Figure 1, peripheral blood: p-value < 0.0001; Peritoneal fluid: p-value < 0.0001). In addition, the significant differences of MMP-9 expression was found between eutopic endometrial tissues. Among them, the eutopic endometrium of endometriosis I–II, endometriosis III–IV and control group were (mean 1.669 ± 0.05226), (mean 1.587 ± 0.05226), and (mean 1.000 ± 0.03181), respectively (Figure 2, Eutopic endometrium: p-value = 0.0002). Therefore, the expression level of MMP-9 was also analyzed statistically for correlation with the clinical stage of endometriosis. An association was observed between MMP-9 gene expression and rASRM classification in the endometriosis group.

Discussion

Despite the fact that research about the biomarkers of endometriosis is continuously increasing, no satisfactory result is found, which makes it impossible to obtain effective laboratory detection used in the diagnosis and monitoring of the treatment of the disease.[16,17] Recently, inflammatory biomarkers have become the subject of interest in the context of endometriosis.[1,14] However, the clinical significance and biological mechanism of inflammatory biomarkers in the development of endometriosis remain largely unknown.[7,17] The study showed the differences in the expression of inflammatory biomarkers between the studied groups of patients that could potentially promote the development of the disease.[18] Since endometriosis was known as a precursor

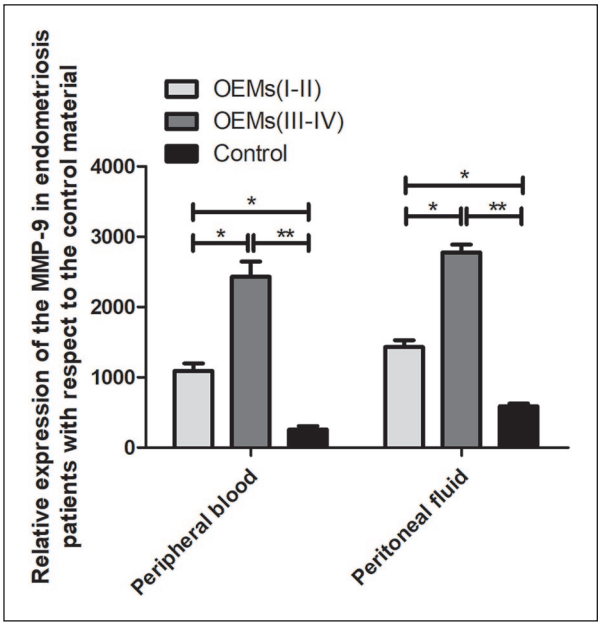

Figure 1. Relative expression of the MMP-9 in the peripheral blood and peritoneal fluid of these endometriosis patients with respect to the control material.
OEMs: ovarian endometriosis.
*p < 0.05, **p < 0.01.

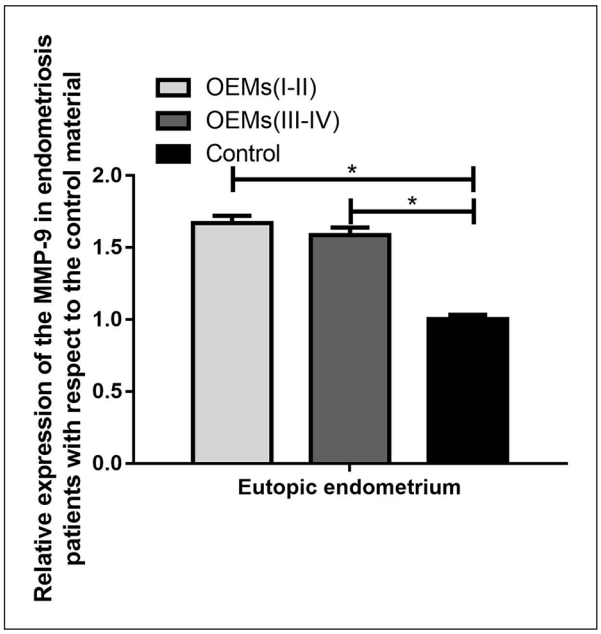

Figure 2. Relative expression of the MMP-9 in the eutopic endometrial tissues of these endometriosis patients with respect to the control material. (OEMs: Ovarian endometriosis).
OEMs: ovarian endometriosis.
*p < 0.05.

to some types of ovarian cancer, similar biological functions of the inflammatory biomarkers detected in cancer might also determine the development of endometriosis.[19,20]

Due to the small number of studies on the expression analyses of inflammatory biomarkers in patients with endometriosis, the effect of the research makes a significant contribution to expanding knowledge about the impact of genetic factors on the development of endometriosis.[1,3,17] Understanding the relationship between gene expression and the risk of endometriosis might contribute to the development of new therapeutic strategies in the context of this disease.[11] Panir et al.[21] showed that unraveling the significance of ncRNAs in endometriosis would pave the way for new diagnostic tests and identify new therapeutic targets and treatment approaches that had the potential to improve clinical options for women with this disabling condition.[11] In addition, Suryawanshi et al.[14] suggested that our study reported for the first time that distinct plasma miRNA expression patterns might serve as highly specific and sensitive diagnostic biomarkers to discriminate between healthy, endometriosis, and endometriosis-associated ovarian cancer cases.[11] There are reports in the scientific press suggesting a link between the expression levels of biomarkers and the development of endometriosis.[3,12] This study aimed to analyze the level of expression of three inflammatory biomarkers (IL-6, VEGF, and MMP-9) in the progression of endometriosis.

In the context of the expression levels of inflammatory biomarkers, the followings were involved: Age, BMI, Gravidity, Parity, Menstrual frequency, Menstrual time, and the clinical stage of endometriosis. An interesting aspect of such an assessment was whether inflammatory biomarkers could serve as a biomarker in a group of patients affected by endometriosis. On this basis, it is possible to identify patients belonging to the risk group whose prognosis would be worse, and the therapeutic process should be more aggressive-even in the case of endometriosis at an early clinical stage. In our study, these three sequences of inflammatory biomarkers (IL-6, VEGF, and MMP-9) we studied were found to be statistically insignificant from the point of view of endometriosis risk. Our results are in contrast to the literature data that support the role of these inflammatory biomarkers in the risk of endometriosis. There are study results showing that the expression of inflammatory biomarkers IL-6, VEGF, and MMP-9 in the ectopic endometrial tissues of endometriosis is much higher than in normal endometrial tissues.[3,12]

The only inflammatory biomarker in which our statistical analysis showed a significant correlation with endometriosis was MMP-9. In the cases of disease, a reduced level of MMP-9 expression was shown. In addition, MMP-9 level correlated with the clinical staging of endometriosis. Compared with healthy controls, MMP-9 expression level was higher in patients with endometriosis, and MMP-9 expression level increased further as the disease progressed. Serum level of MMP-9 showed the significant correlation in these patients with stages III–IV, compared with these patients with stages I–II. Based on these data, it could be concluded that the upregulation of MMP-9 was involved in

the pathogenesis of endometriosis and might be a promising diagnostic and prognostic biomarker of this disease.

With all of the above findings in mind and realizing the limitations of our study, we dare say that this research has shed new light on inflammatory biomarkers in endometriosis and contributes to a growing—but still unclear—knowledge of these sequences of inflammatory biomarkers in endometriosis.[4,20] First, the results presented in this article on the analysis of inflammatory biomarkers reveal the existence of certain relationships of their expression with endometriosis. However, it should be noted that the presented research covers a small sample size and requires further work carried out on much larger groups of respondents. The study groups we use might simply be quantitatively unsatisfactory to draw the right conclusions. Second, the cases and controls are not exactly homogeneous; to some extent, the differences in age, spontaneous abortion, and the use of hormone replacement therapy can result into the bias of results. What is more, the control group is not a group of disease-free women, but they were all treated surgically for a mild gynecological condition-symptomatic uterine fibroids. Finally, some reports suggest that some inflammatory biomarkers may exhibit a correlation with uterine fibroids' tissue as such, yet in our study, the genetic assays were performed strictly on the selected endometrial tissues and not on uterine fibroids.

In view of the relevance of the results obtained, these studies should be continued. The results obtained in the work contribute to the broadening of knowledge on the subject of molecular mechanisms conducive to the development of endometriosis. Gene expression testing could be a future target for personalized therapy. The current state of knowledge about inflammatory biomarkers in endometriosis is still limited. Further research is warranted to explore this topic.

Conclusions

In summary, this study found that these patients with endometriosis showed the significant differences in MMP-9 expression in peripheral blood, peritoneal fluid, and eutopic and ectopic endometrial tissues as the condition worsens. The research suggested that the determination of MMP-9 in peripheral blood has certain value in evaluating the condition of endometriosis, which might play an important role in the pathogenesis of endometriosis and be explored for postoperative recurrence monitoring.

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Author contributions

Maofang Hua wrote the manuscript. Yuan Wang collected case data. ShuangHua Yu and Fanfei Meng edited and corrected the article and provided article ideas. Yuan Wang edited the article for language. Jun Yao contributed to pathological analysis. Zhenxuan Zhu was responsible for obstetrical evaluation. Xiaoyun Liu provided epidemiologic data and made revisions according to the recommendation. All authors commented on and approved the manuscript.

Data availability statement

Data are available from the corresponding author upon reasonable request.

Declaration of conflicting interests

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Ethics approval

Ethical approval for this study was obtained from *the Bioethical Committee of Lianyungang maternal and child health hospital (APPROVAL NUMBER/ID: LYG-ME202004)*.

Informed consent

Written informed consent was obtained from all subjects before the study.

Trial registration

Not applicable.

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