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Granzyme B levels and granzyme B polymorphisms in peripheral blood of patients with endometriosis: a preliminary study

Mine Islimye Taskin, Gurhan Guney, Ertan Adali, Adnan Adil Hismiogullari, Yavuz Dodurga, and Levent Elmasc

ABSTRACT
The chronic course of endometriosis suggests that the immune system may play a role in its aetiology. There may be resistance to cell lysis, as well as an immune defect underlying endometriosis. Granzyme B is a serine protease that is secreted by Natural Killer (NK) cells and cytotoxic T lymphocytes during a cellular immune response and can induce apoptosis. The aim of this study was to evaluate the relationship between both Granzyme B levels and Granzyme B gene polymorphisms in endometriosis patients. Women between the ages of 20–45 were included in the study. The patients were divided into two groups: those diagnosed with endometriosis and those who had not been diagnosed with endometriosis. In the blood samples, Granzyme B gene polymorphisms and serum levels of Granzyme B were studied. There was no difference between the groups in terms of median Granzyme B levels and the presence of AA, AG, and GG genotypes. There was a difference in median granzyme levels for the control group; the GG genotype was found at a lower frequency. The immune defect within endometriosis-related immune cells may not be exclusively due to Granzyme B. Other mediators that are secreted from immune cells may have additive effects.

IMPACT STATEMENT
- **What is already known on this subject?** NK cells are cytotoxic and inhibit the implantation of autologous endometrial cells that are spilled into the peritoneum by retrograde menstruation. Thus, a reduction in NK cell activity may facilitate the progression of endometriosis. The literature review reveals that there are studies suggesting that NK cell activity may be insufficient in endometriosis. Granzyme B is a serine protease that is secreted by NK cells and cytotoxic T lymphocytes during a cellular immune response.
- **What do the results of this study add?** Granzyme B is one of the cytotoxic granules in NK and cytotoxic T lymphocyte cells and its genetic polymorphisms were tested in endometriosis. We found that median Granzyme B levels were significantly different in patients with the GG genotype in the control group, compared to those with the AA and AG genotype. However, this difference was not detected between the control and endometriosis groups.
- **What are the implications of these findings for clinical practice and/or further research?** Our results contribute to uncovering the pathogenesis of endometriosis since there are no previous studies in the literature regarding this topic. Although we did not find a difference, our results will inform further studies made on this topic. Studies with different molecules and an increased number of patients are needed. The immune defect of endometriosis may not be due exclusively to Granzyme B. Other mediators that are secreted from immune cells may have mutual effects and interactions.

Introduction
Endometriosis is a debilitating gynaecological disease affecting millions of women worldwide. Although its pathogenesis has still not been clearly elucidated, it has been explained that it originates from endometrial fragments escaping to the peritoneal cavity during retrograde menstruation. Today, this is one of the widely accepted explanations for the mechanism underlying endometriosis (Ahn et al. 2017). However, since all women with retrograde menstruation do not have endometriosis, the retrograde menstruation theory cannot explain all the cases of endometriosis (Zondervan et al. 2018). Another theory proposed to clarify the unexplained elements of retrograde menstruation is the theory of immunity. The chronic course of endometriosis and the presence of autoantibodies in patients with endometriosis suggest that the immune system may play a role in the aetiology. There might be a resistance to ectopic endometrial cell lysis, as well as an immune defect present in endometriosis (Walankiewicz et al. 2018).
Endometriosis is associated with an abnormal immune response to endometrial cells, which can facilitate the implantation and proliferation of ectopic endometrial tissues. Since endometrial cells mimic cancer cells in their infiltration, proliferation, and adhesion ability within the ectopic regions, natural killer (NK) cells are often theorised as being involved in endometriosis. NK cells are cytotoxic and inhibit the implantation of autologous endometrial cells spilled into the peritoneum by retrograde menstruation. Thus, a reduction in NK cell activity may facilitate the development of endometriosis (Drury et al. 2018).

Granzyme B is a serine protease that is secreted by NK cells and cytotoxic T lymphocytes during a cellular immune response, and it behaves in a similar manner as perforins; it opens holes in the cell and discharges the cytosol with lysis, followed by apoptosis (Voskoboinik et al. 2015; Jin et al. 2019).

The literature review reveals that there are studies suggesting that NK cell activity may be insufficient in endometriosis. However, there has been no method so far to demonstrate this insufficiency (Kralickova et al. 2018). When we referred to the literature, NK cell activity was usually evaluated with indirect methods. Because NK cytotoxicity is mediated by the directed exocytosis of cytolytic granules like perforins and granzymes, which perforate the target cell plasma cell membrane and trigger apoptosis (Campbell and Hasegawa 2013). It has been emphasised in some studies that genetic changes may have an effect on the etiopathogenesis of endometriosis (Deiana et al. 2019). Therefore, these genetic changes may cause Granzyme B gene polymorphisms, and these changes may impair the ability of NK cells or cytotoxic T lymphocytes to destroy endometriotic cells that have spilled into the peritoneum. Based on these facts, we aimed to show that there might be a relationship between both Granzyme B levels and Granzyme B gene polymorphisms in endometriosis. We believe that our results will contribute positively to uncovering the pathogenesis of endometriosis, since there are no previous studies in the literature regarding this relationship.

Materials and methods

This case-control study was performed at Balikesir University School of Medicine, Health Practice and Research Hospital (Balıkesir, Turkey). The study was approved by the ethics committee of the same university. Women admitted between the ages of 20 and 45 to our gynaecology and obstetrics clinic from March 2018 to April 2019 were included in the study. Written consent was obtained from all of the patients. The inclusion criteria were determined as follows: 20 – 45 years of age, confirmation of endometriosis during the operation, and written consent. The exclusion criteria were determined to be chronic inflammatory or systemic diseases, autoimmune diseases, malignancy, and receiving hormone therapy. The patients were divided into two groups: those diagnosed with endometrioma or endometriosis who had received laparoscopic surgeries (n = 50) and those who had received surgery for benign causes (such as follicle cysts, lutein cysts, serous cystadenoma) (n = 36) other than endometrioma. The age, body mass index (BMI), gravida, parity, smoking habit, and symptoms of the patients were recorded. In the blood samples of these two patient groups, Granzyme B gene polymorphisms and serum Granzyme B levels, as well as cancer antigen 125 (CA 125) and C-reactive protein (CPR) levels, were studied. Blood samples were taken soon after the operation to confirm the presence of endometriosis. Postoperative endometriosis was also confirmed pathologically. In the control group, the pathological diagnoses were follicle cysts, lutein cysts, or serous cystadenoma.

Biochemical analysis

The serum samples were separated by centrifuging at 825 g for 10 min for analyses of Granzyme B. The serum levels for this parameter were determined by an enzyme-linked immunosorbent assay using commercially-available kits (Elabscience Company, Wuhan, China) on a diagnostic instrument i.e. the Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, Massachusetts, USA).

Genomic DNA isolation from venous whole blood samples

To isolate the genomic DNA, we collected 2 mL of venous whole blood samples in hemogram tubes from the control and patient groups. The genomic DNA of both groups was isolated using a QIAamp DNA blood kit (Qiagen, Germany) according to the manufacturer’s protocol. The concentration and quality of DNA samples were measured spectrophotometrically via a NanoDrop spectrophotometer (Thermo, USA).

SNP genotyping of the granzyme B gene

To determine Granzyme B rs8192917 (G > A) single nucleotide polymorphisms, LightSNiP assay (TIB Molbiol, Germany) and genotyping were performed with a LightCycler 480 real-time PCR system (Roche, Germany) with regard given to the melting curve analysis. The LightCycler 480 instrument method was carried out as follows: denaturation for 10 min at 95°C; following 45 cycles including denaturation for 10 s at 95°C, annealing for 10 s at 60°C, and extension for 15 s at 72°C; one cycle of melting curve step for 30 s at 30°C, two minutes at 40°C, and 0 min at 75°C (Acquisition Mode: Continuous); cooling stage at 40°C for 30 s.

Statistical analyses

The data analysis was performed by using the IBM SPSS Statistics Version 17.0 software (IBM Corporation, Armonk, NY, USA). The descriptive statistics for the continuous variables were shown as the mean±SD or median (minimum – maximum), where appropriate. Meanwhile, the mean differences between the controls and cases were compared using the Student’s t test, and the Mann-Whitney U test was applied for the comparisons of the variables that were not distributed normally.
Results

The mean ages of the groups were similar ($p = .171$). There was no statistically significant difference between the groups in terms of the median gravidity and parity number, BMI, smoking history, and the CRP levels. The median CA 125 level of the case group was statistically higher than that of the control group ($p < .001$). There was no statistically significant difference between the groups in terms of median Granzyme B levels ($p = .753$) (Table 1).

There was no association between the disease and cases with the AA genotype, AG genotype, and GG genotype ($p = .512$, $p = .380$, $p = .691$, respectively). In addition, there was no association between the disease and cases with the A or G allele ($p = .729$).

When we look at the correlation analyses, no statistically significant correlation was observed between the age, gravidity, parity, BMI, and CA 125 and CRP levels, respectively, in Granzyme B measurements among all cases ($p > .05$). In the control and case groups, no correlation was observed between the age, gravidity, parity, BMI, CA 125 and CRP levels, respectively, in the Granzyme B measurements ($p > .05$) (Table 2).

### Table 1. Demographical and clinical characteristics.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Controls (n = 36)</th>
<th>Cases (n = 50)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>30.0 ± 6.5</td>
<td>30.4 ± 6.8</td>
<td>.171†</td>
<td></td>
</tr>
<tr>
<td>Gravida</td>
<td>0 (0–4)</td>
<td>1 (0–5)</td>
<td>.066‡</td>
</tr>
<tr>
<td>Parity</td>
<td>0 (0–2)</td>
<td>0 (0–2)</td>
<td>.291‡</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.8 (20.3–29.0)</td>
<td>23.8 (17.7–33.3)</td>
<td>.401‡</td>
</tr>
<tr>
<td>Smoking habit</td>
<td>9 (25.0%)</td>
<td>14 (28.0%)</td>
<td>.950‡</td>
</tr>
</tbody>
</table>

### Table 2. The results of correlation analyses between the measurements of granzyme and demographical, clinical characteristics.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Controls (n = 36)</th>
<th>Cases (n = 50)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infertility</td>
<td>–</td>
<td>19 (38.0%)</td>
<td></td>
</tr>
<tr>
<td>Bleeding</td>
<td>–</td>
<td>5 (10.0%)</td>
<td></td>
</tr>
<tr>
<td>Pelvic pain</td>
<td>–</td>
<td>26 (52.0%)</td>
<td></td>
</tr>
<tr>
<td>CA 125</td>
<td>11.5 (3.0–30.0)</td>
<td>38.0 (4.0–165.0)</td>
<td>&lt;.001‡</td>
</tr>
<tr>
<td>CRP</td>
<td>3.3 (3.1–9.0)</td>
<td>3.3 (1.2–39.0)</td>
<td>.154‡</td>
</tr>
</tbody>
</table>

### Table 3. The measurements of granzyme levels regarding for both groups and genotypes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls (n = 39)</th>
<th>Cases (n = 50)</th>
<th>p Value</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (control = 20) (cases = 23)</td>
<td>11.8 (9.2–28.4)</td>
<td>12.0 (9.3–17.6)</td>
<td>.884</td>
<td>11.9 (9.2–28.4)</td>
</tr>
<tr>
<td>AG (control = 13) (cases = 24)</td>
<td>13.9 (9.4–22.9)</td>
<td>12.0 (8.3–20.9)</td>
<td>.179</td>
<td>12.7 (8.3–22.9)</td>
</tr>
<tr>
<td>GG (control = 3) (cases = 3)</td>
<td>9.4 (9.2–10.8)</td>
<td>13.0 (10.1–16.1)</td>
<td>.200</td>
<td>10.5 (9.2–16.1)</td>
</tr>
</tbody>
</table>

In cases with the AA genotype, there was no statistically significant difference between the control and case groups in terms of the median Granzyme B levels ($p = .512$). In addition, there was no difference between the control and case groups in terms of median Granzyme B levels in cases with the AG and GG genotypes ($p = .380$ and $p = .691$).

Patients carrying the GG genotype have lower levels of Granzyme B than patients carrying the AA and AG genotypes in the control group ($p = .006$ and $p < .001$). There was no difference between the AA and GG genotypes in terms of the Granzyme B levels, according to the Bonferroni Correction ($p = .028$). No statistically significant difference was found in the median Granzyme B levels based on the genotypes in the case group ($p = .840$) (Table 3).

Discussion

In this study, the Granzyme B serum levels and the Granzyme B genetic polymorphisms were compared between the case and control groups, and no significant difference was found. Although the Granzyme B level was found to be higher in patients with the GG genotype, a statistically significant difference was not found.

Granzyme B is a protease belonging to the chymotrypsin family and is a component of the cytolytic granules of cells. Granzyme B plays an important role in pathologies such as anti-tumour immunity, viral infections, autoimmunity, and graft-versus-host disease (Trapani and Sutton 2003). Different polymorphisms in the Granzyme B gene can affect the production, degranulation, and cytotoxicity of Granzyme B (Zhang et al. 2018; Mhaidat et al. 2014). In a cohort study of newly-diagnosed breast cancer patients, the presence of RAH alleles in the Granzyme B gene was found to increase the risk of developing breast cancer, compared to QPY alleles. Considering that endometriosis has some common features with cancer, the importance of the investigation of Granzyme B polymorphisms in this disease will improve the understanding of cancer and endometriosis mechanisms (Gaafar et al. 2009). In another study, Yentur et al. (2014) stated that Granzyme B polymorphisms could facilitate the development of subacute sclerosing panencephalitis by reducing the capacity of NK cells to kill measles-infected cells. Bianco et al. (2012) concluded that as a result of their research, various polymorphisms in the immune system may cause changes in the immune system hemostasis, causing ectopic endometrial $foci$ to survive and enable disease progression. Investigating the mechanisms behind how these polymorphisms cause
endometriosis will illuminate the pathophysiology of the disease.

In our study, we found that median Granzyme B levels were significantly different, statistically, in patients with the GG genotype in the control group, compared to those with AA and AG genotypes. However, when we look at the differences between the groups, we found that there was no significant difference in Granzyme B levels. Although the level of Granzyme B was higher in patients with the GG allele, there was no statistically significant difference found. If we had more subjects, this difference could have been statistically significant. As a result, we have learned that large-scale studies are needed with more subjects.

Although most studies have shown that there is no difference in the number of peripheral NK cells in endometriosis patients, some studies have shown that women with endometriosis have local and systemic changes in the phenotype and functions of NK cells, and these changes manifest themselves in a reduction of cytotoxic function in the peripheral and peritoneal NK cells (Matsuoka et al. 2005; Oosterlynck et al. 1994; Kanzaki et al. 1992). For example, Oosterlynck et al. (1992) and Tanaka et al. (1992) have shown that both the number of peripheral NK cells and their cellular activity in endometriosis decreased. NK cell cytotoxic activity in endometriosis has been performed on K562 human leukaemia cells serving as a target (Oosterlynck et al. 1992). Oosterlynck et al. (1994) have concluded that the decreased NK activity reported in peripheral blood and peritoneal fluid women with endometriosis was not likely to be caused by a quantitative defect, since the percentage of NK positive lymphocytes was not different between women with and without endometriosis.

It has been stated that changes in NK cell activity may correlate with the severity of endometriosis in some studies. For example, in a study on patients with endometriosis active in only the rectosigmoid region, there was an increase in the number of peripheral NK cells when compared to normal fertile controls (Dias et al. 2012). Although our patients had moderate or severe endometriosis, we could not detect any significance of the NK product Granzyme B. This is one of the limitations of our study; it was not set up to classify the severity of endometriosis cases. There is another limitation that came from studying only one of the NK cell products, Granzyme B. This is an indirect evaluation method for NK cell function. It is clear that further research is needed to investigate other molecules with cytotoxic effects different from Granzyme B in terms of NK cell function. Although our hypothesis is that Granzyme B levels should have decreased in endometriosis, we did not find any evidence to support this in the peripheral blood. Additionally, if we had evaluated the peritoneal fluid or endometrioma cysts, we could have found a significant difference.

Endometriosis is a chronic disease characterised by remission and relapses. Our endometriosis patient group at the time of taking samples could have been in the remission period, when inflammation is suppressed (Ścieżyńska et al. 2019). One finding supports this remission period; it found that CRP levels are similarly low between the control group and the endometriosis group in this study. When we discussed the lack of difference found in our study, we touched on the fact that some unknown substances in the sera of the patients with endometriosis may make peripheral NK cells less cytotoxic. This clarifies why the endometrial tissue that has been spilled into the peritoneum is difficult to clean. When the peritoneal fluid of women with endometriosis is confronted by NK cells, their cytotoxicity decreases (Thiruchelvam et al. 2015). In a current study, it was shown that an increased level of interleukin-6 (IL-6) in the peritoneal fluid of patients with endometriosis suppresses NK cell cytolytic activity by down-regulating cytolytic granule components, such as Granzyme B and perforin (Kang et al. 2014). In another study, authors showed that IL15 derived from endometrial stromal cells stimulates the growth and invasion of endometrial stromal cells. In addition, IL15 may help the immune escape of endometrial stromal cells by suppressing the cytotoxic activity of NK cells in the ectopic milieu, thereby facilitating the progression of endometriosis (Yu et al. 2016). If we studied Granzyme B in peritoneal fluid and different cytokine levels, these additional details could show decreased cytotoxicity in endometriosis. In a recent review, it was discussed that the decrease of NK cell cytotoxic activity in endometriosis may be associated with an increased expression of some inhibitory NK cell receptors and also may be due to inhibitory cytokines present in the peritoneal milieu of patients with endometriosis (Ścieżyńska et al. 2019). This may also be the reason the lack of difference found in our study.

Granzymes are serine proteases that are produced by NK cells and cytotoxic T lymphocytes (Akbari et al. 2017). Five Granzymes have been recognised in humans; Granzyme B is the most potent enzyme and exhibits the most severe anti-tumour and apoptotic activity in tumour cells or virus-infected cells. In our study, Granzyme B levels in the peripheral blood can also be affected by the cytotoxic T lymphocytes and their different activity in different patients. When the studies are carefully analysed, it can be observed that there is a decrease in NK function, but there is no consensus on how to explain it. When the possibility of intracellular granules being responsible for cytotoxicity is considered, the reason why research into these granular contents is important becomes apparent. Considering that endometriosis is an oestrogen-dependent disease, the relationship between elevated oestrogen levels and increased levels of chemottractants in the peritoneal fluid may be understood. In studies, it was observed that peritoneal NK cell numbers were not different in patients with endometriosis (Oosterlynck et al. 1994). Increased NK cell chemotactants with increased potential NK cell counts were thought to be counterbalanced by increased NK cell death (Simoni and Taylor 2018). In our patients, this may be the reason we could not find any differences in Granzyme B levels as an indirect indicator of NK cell activity. It is possible that Granzyme B levels may initially increase and decrease afterwards, depending on cell death, and this then appears in the laboratory as unchanged levels.

Uterine NK (uNK) cells are a different NK cell population that has been recently proposed. In this phase of the menstrual cycle, the number of uNK cells markedly increase and differentiate from the haematopoietic stem cells by migration...
from the peripheral blood. During early pregnancy, uNK cells form a dense infiltrate around the trophoblast cells, and this continues with placentation. Therefore, uNK cells are considered important for a successful pregnancy and constitute the basis of infertility studies. When this relationship with fertility is analysed, it is quite clear that it may play a role in the pathogenesis of endometriosis. The investigation of the relationship between uNK cells and peripheral uterine NK cells in the pathogenesis of endometriosis in a prospective randomised trial will further explain the aetiology of endometriosis (Mariee et al. 2012; Seshadri and Sunkara 2014; Chen et al. 2017).

In our study, the hypothesis that the resistance to cell lysis after retrograde menstruation in women with endometriosis related to one of the cytotoxic granules, Granzyme B, was tested, but no significant difference was found. In the current literature, since the role of peripheral NK cells in endometriosis cannot be clearly demonstrated, studies with different molecules and an increased number of patients are needed. As a result, the immune defect seen in endometriosis may not be due exclusively to Granzyme B. Other mediators that are secreted from NK and other immune cells may have mutual effects and interactions.

**Disclosure statement**

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**ORCID**

Mine İslimye Taskın http://orcid.org/0000-0001-9199-1679

Gurhan Gunes http://orcid.org/0000-0002-0093-2743

Ertan Adali http://orcid.org/0000-0003-3031-1646

Adnan Adil Hismiogullari http://orcid.org/0000-0001-7459-6319

Yavuz Dodurga http://orcid.org/0000-0002-4936-5954

Levent Elmas http://orcid.org/0000-0002-6865-6666

**References**


Drury JA, Parkin KL, Coyne L, Giuliani E, Faizzeabas AT, Hapangama DK. 2018. The dynamic changes in the number of uterine natural killer cells are specific to the eutopic but not to the ectopic endometrium in women and in a baboon model of endometriosis. Reproductive Biology and Endocrinology 16:67.


expressing lymphocytes in endometriosis: correlation with clinical and laboratory parameters. Mediators of Inflammation 2018:1.


