



Immunohistochemical Evaluation of NKP46 Receptor Expression and the Number of NK Cells in the Endometrium of Patients with Endometriosis

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ABSTRACT

Background: Endometriosis is a medical condition that can cause infertility in women. Women with endometriosis experience a decrease in NK cell cytotoxic activity against endometrial cells, ultimately contributing to the spread of these cells.

Objective: To assess the frequency of NK cells and the expression of the NKP46 receptor in endometrial tissue from patients with endometriosis using immunohistochemistry.

Methods: 30 endometrial tissue specimens were collected from three groups of cases with mild (n=11), moderate (n=10), and severe endometriosis (n=9), respectively. Additionally, 20 normal endometrial tissue specimens were collected as the control group. Immunohistochemical staining was carried out using specific human monoclonal antibodies against CD56 and NKP46 molecules.

Results: Cases with severe endometriosis had a significantly higher number of CD56⁺ uterine NK cells (26.19±2.50) compared to fertile women (15.02±0.622) and women with mild to moderate endometriosis ($p<0.001$). However, there was no significant difference between the mild to moderate patients compared with the healthy women ($p>0.05$). Endometrial NKp46 expression was lower in women with severe endometriosis (0.447±0.0829) compared to fertile women (0.987±0.115, $p=0.03$). The NKp46⁺/CD56⁺ cell ratio was also lower in women with severe endometriosis (0.019±0.003) compared to fertile women (0.072±0.011, $p=0.01$).

Conclusion: Women with severe endometriosis demonstrated an increased rate of infiltrated uterine NK cells and a significant decrease in NKP46 expression compared to fertile women. Therefore, NK cells and the NKp46 receptor may be involved in the development of endometriosis.

Keywords: CD56, Endometriosis, NKp46, Uterine NK Cells

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INTRODUCTION

Endometriosis is a chronic female disease characterized by endometrial tissue and stroma outside the uterus (1). About 10% to 12% of women of pregnancy age and up to 60% of women with fertility problems experience endometriosis (2). Symptoms generally include dysmenorrhea, deep dyspareunia, pelvic pain, and infertility (3). Despite the fact that endometriosis was first identified over 150 years ago, not much is known about its pathogenesis and pathophysiology so far (4). Several theories have explained the pathophysiology of endometriosis. Sampson's theory of retrograde reflux and eutopic endometrial implantation is the most accepted one, although it does not explain why some of the cases with retrograde reflux spread endometriosis (5).

It has been indicated by recent research that the development of endometriosis is closely related to the functioning of the immune system (6). Accordingly, the spread of endometriosis may be affected by several immunological factors such as autoantibodies, non-recognition and clearance of eutopic endometrial cells by the immune system (7). Incomplete immune care in women with endometriosis causes stability and progress of endometriosis tissue outside the uterine (8).

Identifying and eradicating irregular cells constitutes a vital function carried out by the immune cells (9). It has been suggested that a major factor in endometriosis is the deterioration of the immunological response, particularly natural killer (NK) cell activity, which leads to insufficient clearance of refluxed menstrual material (10).

There are NK cell populations in the peripheral blood (PB) and the uterus, primarily identified as CD56^{dim}CD16⁺ and CD56^{bright}CD16⁻, respectively (11). NK cell activity is regulated by the adjustment between the activation and inhibition of the receptors expressed on their surface. The receptors NKp46, NKp44, NKG2D, CD16, CD107a, and CD69 are indicators of the NK cell cytotoxicity (12).

NCR1 or NKp46 is a surface molecule classified as a superfamily of immunoglobulins. It is exclusively found in NK cells and plays a crucial role in initiating natural cytotoxicity. NKp46 is a novel surface molecule and is crucial for triggering the natural cytotoxicity of NK cells. NKp46 is expressed on all NK cell subsets and participates in human NK cell activation. Micro clusters are formed by NKp46 at the immune synapse that exists between target cells and NK cells. NKp46, along with NKp44 and NKp30, is one of the NCRs which play an important role in the lethality of NK cells (13, 14).

The role of NKp46 is imperative in the development of endometriosis. NK cell cytotoxicity defects have been linked to the occurrence and advancement of endometriosis, according to research findings. The expression of NKp46 in endometrial tissues has also been linked to abortion and infertility in women suffering endometriosis (15). These results indicate that NKp46 could be a promising treatment for endometriosis. NKp46 may be involved in recognition and response to ectopic endometrial tissue. The activation of NK cells through NKp46 could lead to the immune-mediated clearance of endometrial lesions or modulate the inflammatory environment correlated with endometriosis (16). However, the precise mechanisms and the overall impact of NKp46 in the pathogenesis of endometriosis need further investigation for a comprehensive understanding. Improved understanding of NKp46 may aid endometriosis prognosis and treatment. Our study aimed to examine the ratio of NK cells in the endometriosis patients, focusing on the expression level of the NKp46 receptor. This research will be trying to provide a better perspective of the capacity of this receptor in endometriosis for specialists and researchers in the field.

MATERIALS AND METHODS

Human Subjects

The biopsies were taken from all women

attending the Obstetrics and Gynecology Department of Imam Khomeini Hospital, Ahvaz, Iran. The samples were collected from the patients who agreed to fill in the informed consent form. Also, the study (IR.AJUMS.MEDICINE.REC.1398.012) has been approved by the Ethical Committee of Ahvaz Jundishapur University of Medical Sciences. Two groups of women were recruited. The first group comprised patients with endometriosis who underwent laparoscopic surgery at Imam Khomeini Hospital. (n=30). The patients were categorized in three groups: mild endometriosis group (n=11), moderate endometriosis group (n=10), and severe endometriosis group (n=9). As a part of their ongoing investigation, a timed endometrial biopsy was taken.

The second group of patients were normal fertile women who underwent curettage in the operating room of Imam Khomeini Hospital with no specific pathological problems (n=20). The patients with metabolic disorders and immune suppression as well as other diseases such as thyroid and lupus disease, uterine infection, uterine cancer and uterine anatomical disorders were excluded from the study. Also, the women had not consumed any immunosuppressive or hormonal drugs at the sampling time. The control and the patient groups were matched for age (all aged 20-45). Also, the women in the healthy control group had no history of abortion with at least one successful pregnancy history (Table 1).

Tissue Collection

On days 21 and 24 of the menstrual cycle, a routine endometrial biopsy was performed on each participant. To perform immunohistochemistry, endometrial tissue

specimens were treated with 10% formalin as the fixator for 24 h. They were transferred to the pathology laboratory as soon as possible and paraffin-embedded blocks were maintained until immunohistochemistry was performed.

Immunohistochemistry

The CD56 and NKP46 marker expression in all the endometrial samples was assessed by immunohistochemical staining. Endometrial biopsy specimens underwent a series of steps in this procedure, including immersion in 10% neutral-buffered formalin fixed for a full day, graded ethanol was used for dehydration, followed by xylene for clearing, and finally, paraffin wax was used for embedding. Using the Leica RM2235 microtome, slices 4-5 μm thick were cut from the paraffin-blocked tissues. The tissue sections were placed on glass slides coated with 3 Triethoxysilane-propylamin (Sigma Chemical Co., Poole, UK). They were then rehydrated using decreasing concentrations of alcohol after being dewaxed in xylene. Using an 800W microwave for 25 min, antigen-retrieval procedure was used to retrieve the antigen (17). Following a wash in PBS buffer and distilled water, the tissue slices were incubated with protein blocker solution (Biopharmadx, Germany) for NKP46 and CD56 for one and ten min, respectively. Subsequently, the primary monoclonal antibodies were respectively applied for CD56 (diagnostic bio-systems, Pleasanton, CA, USA) for 90 min at 37°C as well as for NKP46/NCR1 (3 $\mu\text{g}/\text{mL}$; R&D Systems, Minneapolis, MN, USA) overnight at +4°C in a humidified chamber. The secondary anti-IgG mouse or anti-goat antibody was used to discriminate the bound antibodies

Table 1. Demographic data of study population

Groups	Control	Mild	Moderate	Severe	p.v
N	20	11	10	9	N.S
Age	43.6±5.85	31.8±6.16	29.6±5.58	32.11±4.48	
Pregnancy rat	100%	27%	10%	0%	
Ens Family history	-	18.2%	30%	22.2%	
Dysmenorrhea (%)	-	54.5%	60%	100%	

for 1 h at 37°C. The next step involved the peroxidase reaction, accomplished by using DAB chromogen (3,3'-diaminobenzidine tetrahydrochloride; Biopharmadx, Germany). After the dyeing process, the colored sections were further stained with hematoxylin and then dehydrated using increasing concentrations of alcohol, and mounted with Entellan rapid non-aqueous medium of polymers in xylene (Merck Millipore.; German), and then tested by optical microscopy. Immunostaining technique reliability was evaluated using positive specimens of NKp46/NCR1 and CD56⁺ lymphoma tissue. Instead of using primary antibodies, PBS buffer was used as a false negative control.

The staining of specimens was evaluated using an Olympus BH2 microscope (Olympus, Melville, NY, USA). Ten images with high-resolution at 400 magnifications were counted for each specimen. The ratio of CD56 positive or NKp46 cells (brown stain)

was calculated against the total endometrial cells (blue stain).

Statistical Analysis

The data were analyzed using SPSS version 22. The distribution of the data was analyzed with the Kolmogorov-Smirnov test and reported as mean±SD. Parametric tests were utilized for data analysis because the data were normally distributed. One-way ANOVA test was used to compare the data between the four groups (the fertile control and the patients; the mild, moderate and severe endometriosis). Post hoc Tukey test was also used for comparison between the control and the patient groups. A *p*-value less than 0.05 was considered statistically significant.

Frequency of CD56⁺ or NKp46⁺ Cells in the Endometrium

The CD56 or NKp46 expression in the endometrial samples was assessed in the

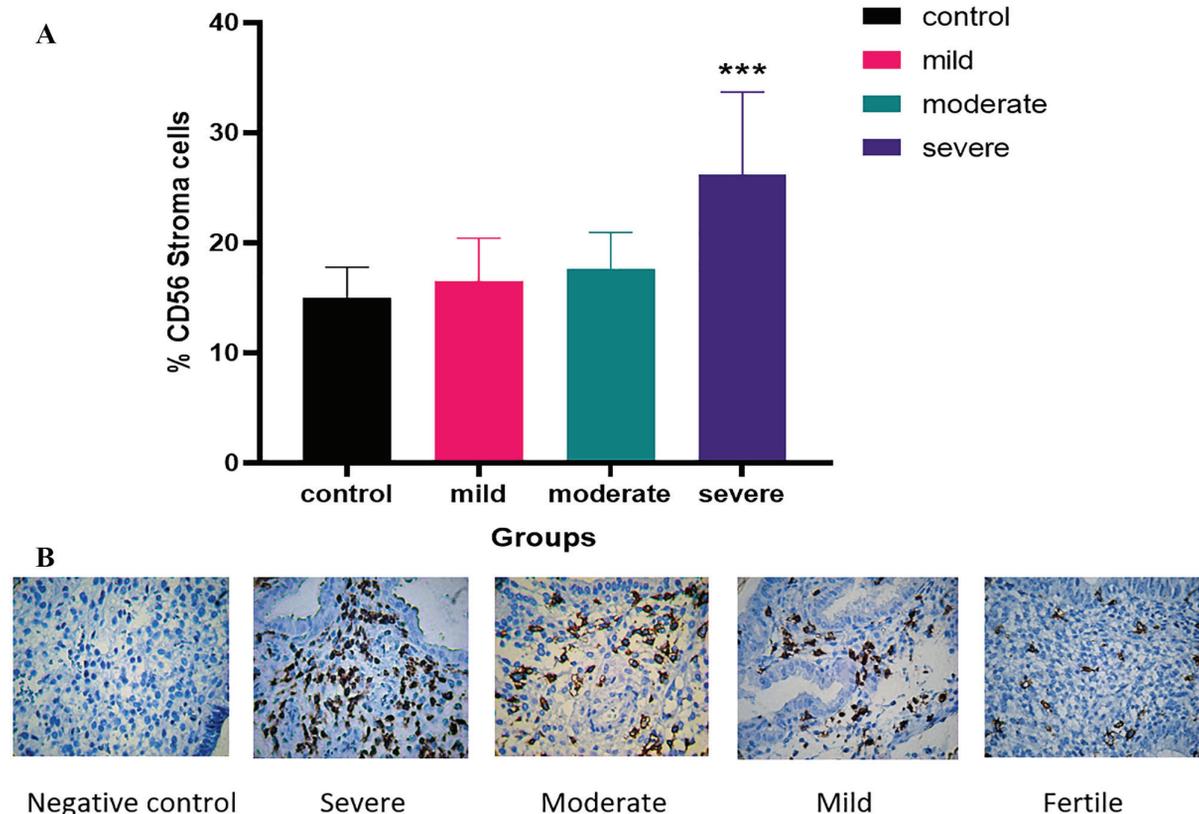


Fig. 1. A) Percentage of CD56⁺ cells in endometrial stroma in three groups of the patients and the control fertile group. B) CD56 expression in the patient groups (mild, moderate and severe) with fertile women as the control. Staining with PBS buffer is also shown for the negative control (400 magnification). CD56 expression significantly increased in the women with severe endometriosis (*p*<0.001) compared with the healthy women and women with mild to moderate endometriosis.

patients with endometriosis and the healthy women using immunohistochemistry. After quantification, it was observed that the cases with severe endometriosis had an elevated ratio of endometrial stroma CD56⁺ cells significantly higher ($26.19 \pm 2.50\%$) than the cases with mild to moderate endometriosis (15.02 ± 0.622 , $p < 0.001$, Fig. 1). It was observed that there is no significant difference between the mild to moderate cases and the fertile women. The NKp46⁺ cell ratio in the stroma was significantly lower in the cases with severe endometriosis (0.447 ± 0.0829) compared with the healthy women (0.987 ± 0.115 , $p = 0.03$). No significant difference was found between the mild to moderate patient compared with the healthy women, Fig. 2). The NKp46⁺/CD56⁺ cell ratio in the endometrial was also calculated, and it was lower in the women with severe endometriosis (0.019 ± 0.003) compared with

the healthy women (0.072 ± 0.011 , $p = 0.01$). However, no significant difference was found between the mild to moderate patient compared with the control group (Fig. 3).

DISCUSSION

The pathogenesis of endometriosis has not been well characterized so far, however the hypothesis that has gained the most widespread acceptance is the occurrence of menstrual bleeding endometriosis and immunologic deficits in its disposal (18). Immune system dysregulation in endometriotic environments is crucial in this respect. It is blurred, therefore, how the immune system leads to the disease's etiology. The uterus contains various immune cells, with the uterine natural killer cell (uNK) being the main immune cell in the uterine endometrium. In the human

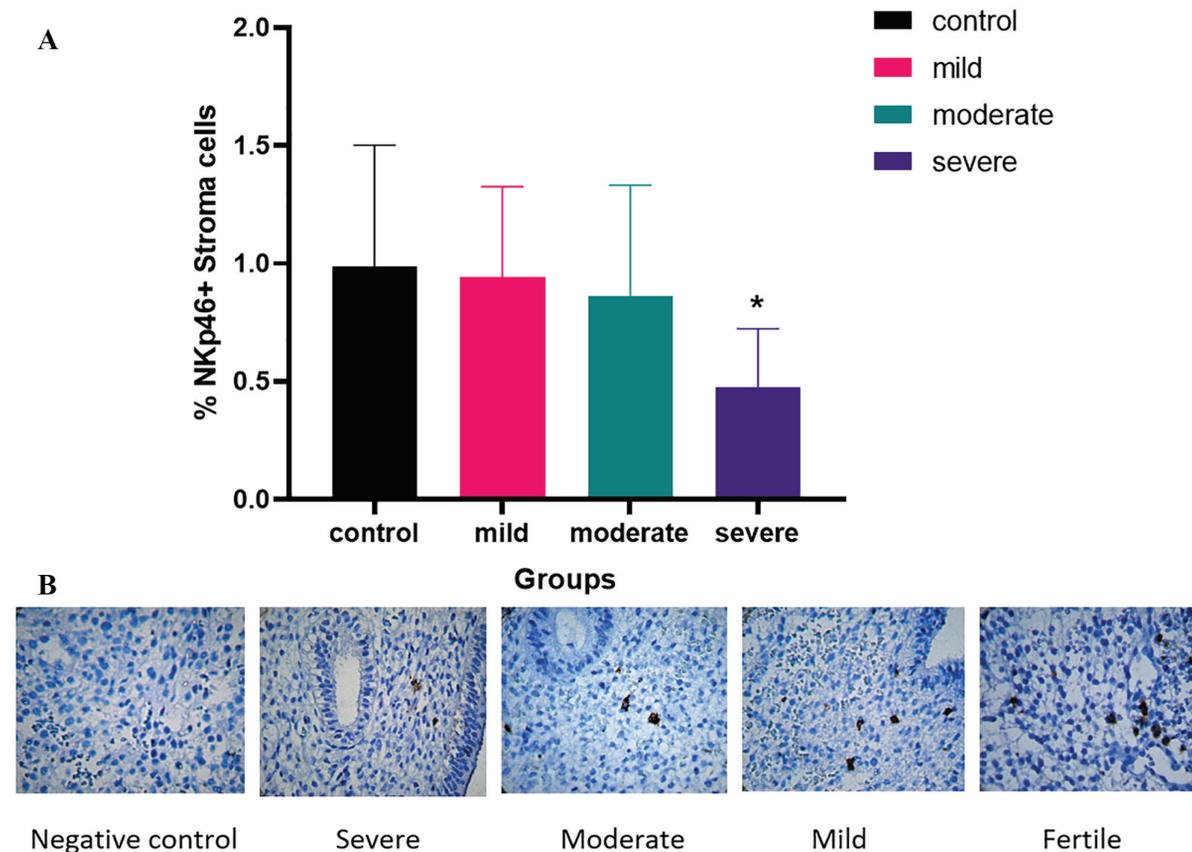


Fig. 2. A) Ratio of NKP46⁺ cells in three groups of patients (mild, moderate and severe) and the control fertile group. B) NKP46 expression in the patient groups (mild, moderate and severe) with fertile women (control). Staining with PBS buffer was conducted for the negative control (400 magnification). NKP46 expression significantly decreased in women with severe endometriosis ($p = 0.03$) compared with the healthy women and those with mild to moderate endometriosis.

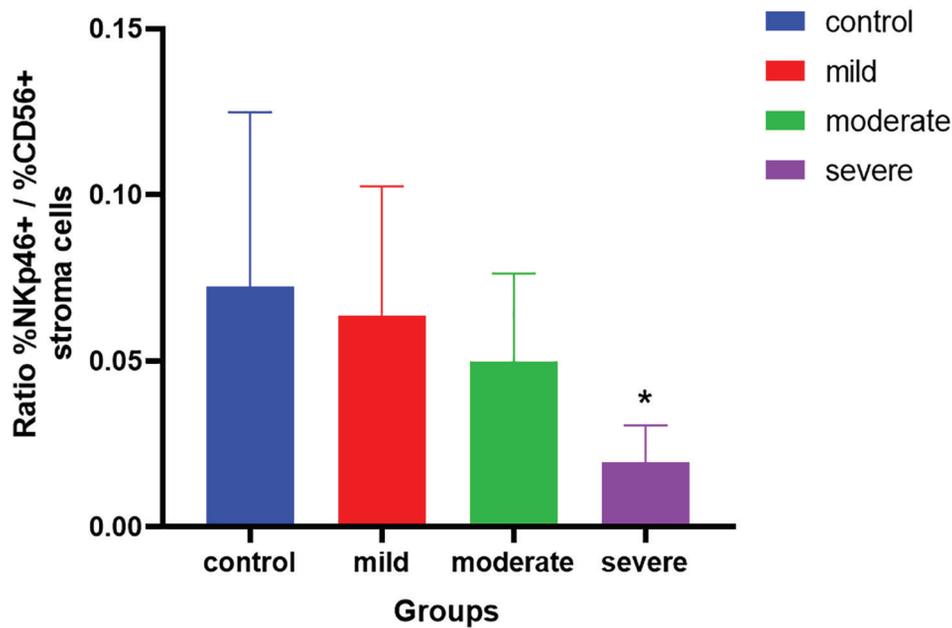


Fig. 3. Ratio of NKp46⁺/CD56⁺ cells in the endometrial stroma of patients with severe endometriosis, with mild to moderate endometriosis and in the healthy women (the control). The ratio of NKp46⁺/CD56⁺ cells was lower in women with severe endometriosis compared with the fertile women ($p=0.01$). However, no significant difference was seen between the mild to moderate patient compared with the fertile women.

endometrium, it plays a pivotal role in modulating the immunological response (19).

We compared the ratio of NK cells between the endometriosis patients and the healthy control group. Our results showed that the cases with severe endometriosis have significantly increased levels of infiltrated CD56⁺ uNK cells compared with the control and other groups including the patients with mild and moderate endometriosis. This result supports a prior study's hypothesis that uNK derangements and endometriosis are related. In fact, Antonio Dias et al. (20) reported that the average ratio of NK cells in women with endometriosis was higher than in the normal group. There is debate among researchers regarding the correlation of an elevating in the ratio of NK cells with severe forms of endometriosis. However, it is believed that this increase is an immunological defense mechanism. As the disease progresses, the immune system tries to restrict the damage caused by the disease by increasing the percentage of NK cells (21). Our study's results also support this perspective. Despite the increase in the NK cell percent in severe forms of endometriosis, interestingly, the

killing activity of these cells decreases in severe forms of the disease (14). Although there is a reduction in the ability of NK cells to kill cells in the PF of women with severe endometriosis, studies have shown that the NK cell ratio and their activation markers (CD69, CD25 and HLA-DR) are similar in cases with endometriosis in comparison with the healthy women (22). Furthermore, severe endometriosis reduces NK cell activity and lethality due to heightened inhibitory killer cell immunoglobulin-like receptor expression and increased HLA-1 expression on NK cell surfaces (23). In the later stages of the endometriosis, the NK cell activity is hampered due to the heightened intensity of inhibitory signals. These signals are responsible for reducing the effectiveness of NK cells, the essential part of the immune system. As a result of NK cell effectiveness reduction, the severity of the disease increases (24).

In contrast, some studies have suggested a reduction in the percentage of NK cells in the patients suffering endometriosis. Freitag et al. (25) found out that endometriosis-affected women do not have a higher probability of increased uNK cells than the control

group. Furthermore, another investigation revealed that the severe endometriosis group had considerably lower percentages of CD56⁺ and CD56^{dim} cells than the control group (26). Various factors such as study design and methodology, disease stage and severity, patient heterogeneity, treatment effects, small sample sizes, geographical and ethnic differences may contribute to the varying findings. For example, variations in the timing of sample collection during the menstrual cycle or the use of different techniques for NK cell measurement could lead to divergent outcomes. The stage and severity of endometriosis can vary among patients. It is possible that NK cell levels fluctuate at different stages of the disease. Researchers need to consider these factors and conduct well-designed, larger-scale studies with standardized methodologies to gain a comprehensive understanding of NK cell roles in endometriosis.

The NCRs have a crucial function in the activity of NK cells (27). The NKP46 receptor is a type I membrane glycoprotein that contains two extracellular C2-type Ig-like domains. It is associated with the tyrosine phosphorylation of FcεRIc and CD3ζ (28). NK cells that are activated express the NKP44 receptor on their surface. However, both NK cells that are inactive and those that are active initially possess surface expression of the NKP46 and NKP30 receptors. Furthermore, the generation of cytokines and NKP46 and NKP30 is essential to the cytotoxic capability of NK cells (27, 29, 30). In the present study, we analyzed the expression of NKP46⁺ cells in the cases with endometriosis and the normal group. The assessment of the results revealed that the expression of NKP46⁺ cells in the endometrium of severe patients group significantly decreased compared with the normal ones. Similarly, Funamizu et al. (26) showed a significant decrease in the ratio of NKP46⁺ NK cells on CD56⁺ NK cells in patients with severe endometriosis compared with the controls. However, Giulian et al. (15) observed that the average proportion of

NKP46⁺ cells in the endometriosis patients and those with unexplained infertility was not significantly different from that in the healthy women. They also calculated the NKP46⁺/CD56⁺ cell ratio in endometrial stroma. The percentage significantly increased in endometriosis cases compared with the fertile group. Furthermore, research has revealed a reduction in the expression of NCR (NKP46 and NKP30) in NK cells found in both PB and PF of endometrial cells. Additionally, there was a decrease in the presence of NKP30 on CD56⁺ NK cells, and NKP46 expression on CD56⁺ NK cells was lower than in the control group (31). This difference is probably related to the type of NK cells in the endometrium and PB. The NK cells found in the PB have an activated phenotype, with most being CD56^{dim}CD16^{bright}. The proportion of CD16⁺ NK cells is resembling that of CD56^{dim}NK cells. These cells are more cytotoxic as they secrete perforin and granzyme. However, in the case of endometriosis, the NK cells existing in the PF and endometrium are CD56^{bright}CD16^{-/low} and have a modulatory phenotype, particularly in the later stages. These NK cells produce cytokines, such as GM-CSF, IFN-γ, IL-10, and TNF-α (32). NK cells in the endometrial environment have limited killing power, but produce a large amount of IFN-γ and TNF-α. This is reflected in their low expression of NKP46. This high cytokine level and reduced NKP46 expression, increase angiogenesis and the advancement of endometriosis (26, 33).

Studies on natural killer cells isolated from uterine endometrium of pregnant women have shown that NKP46 receptor expression on normal killer cells increased as directly related to NK cell cytotoxicity rate. In our study, in addition to the NK cells number, their killing activity was also measured indirectly. NKP46 may regulate cytokine production (IFN-γ, TNF-α, IL-4, IL-10, and TGF-β) in endometrial NK cells. Saeki Shinichiro indicated that although NK cell activating marker NKP46 decreased, the endometriosis patients had a considerably higher ratio of NK cells generating

IFN- γ than the control group. Furthermore, NKp46 expression significantly inversely correlated with type 1 cytokine production, including TNF- α and IFN- γ in NK cells (33). These findings align perfectly with the hypothesis put forth earlier. NKp46 expression on NK cells may lead to reproductive failure due to cytotoxic activity and disruption of cytokine production (14). In their systematic review, José Lourenço Reis et al. investigated the effects of NK receptors on the development of endometriosis. Their findings indicated that overexpression of NK cell inhibitor receptors and a reduction in NK cell activating receptors, such as NKp46, are characteristic of endometriosis pathogenesis (16). Based on all the research conducted, the outcome was predictable. Dysmenorrhea was one of the phenomena that showed a significant increase in the patients with the progress of the disease. Studies have also reported that women who had dysmenorrhea developed endometriosis in the future (34). Based on this, dysmenorrhea is suggested to be a potential risk factor for development of endometriosis. Overall, in this study, we aimed to explore another aspect of the importance of NK cells in the pathogenesis of endometriosis. Undoubtedly, more extensive studies in the future can offer a more complete insight of the role of these cells in the disease.

CONCLUSION

The results of our study revealed that NKP46 receptor expression on NK cell decreased in endometriosis women compared with the healthy group, and that as the disease becomes more severe, the level of expression of NKP46 decreases even more. This suggests that there is a defect in the cytotoxic activity of NK cells in the uterus, involved in the development of endometriosis.

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AUTHORS' CONTRIBUTION

MA conceptualized and designed the methodology. MGH conducted data analysis, prepared the original draft, and supervised the project. FM collected endometrial biopsies. NR performed pathological examination of tissues. MGH and MA used software and validated the data. AM wrote, reviewed and edited the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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