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Nuclear factor-kappa beta pathway and endometrial cancer: a pilot study

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Summary

Objective: Examination of the role of nuclear factor-kappa beta (NF-kB) expression in the etiopathogenesis of endometrial cancer, by means of the immunohistochemical method. *Materials and Methods:* Patients who applied to participate in the study at the clinic were grouped into three categories: those diagnosed with benign endometrial pathology, those with endometrial hyperplasia, and those with endometrial cancer. NF-kB analysis was conducted in the endometrial tissues of the patients' paraffin blocks by means of the immunohistochemical method. For objective assessment purposes, the H score of each patient was calculated. SPSS 15.0 program was employed for statistical analysis. *Results:* The average H score of the first group, comprising benign endometrial pathologies, was 102.4 ± 85.9, that of the hyperplasia group was 143.6 ± 122.4, and that of the cancer group was 276.8 ± 61.8. The average values of groups 1 and 2 were similar ($p = 0.349$); however, the third group's average H score was significantly higher ($p < 0.001$). *Conclusion:* NF-kB, which is a critical mediator in the inflammation process, might be related to the development of premalign and malign endometrial changes.

Key words: Endometrial cancer; Endometrial hyperplasia; Inflammation; NF-kB.

Introduction

Endometrial cancer is the most frequently detected of the gynecological tumors and ranks fourth among female cancers. These statistics serve to illustrate the seriousness of the threat this cancer poses to women's health. At the same time, its exhibition of early clinical symptoms indicates that most cases are diagnosed at an early stage, and a cure is assured if surgical intervention occurs before the disease develops to the advanced stage, minimizing the need for adjuvant radiotherapy and chemotherapy [1]. The presence of unopposed estrogen is now universally accepted as a factor in the etiology of endometrial cancer. Age, parity, and genetic factors are also recognized [2].

Another important factor in the etiology of the disease is endometrial hyperplasia. This pathology can occur at almost any age and mostly causes abnormal uterine bleeding. What makes it special is that endometrial cancer has precursor lesions and/or comes with concomitant malignant cells [3]. The way endometrial hyperplasias are classified was revised by the World Health Organization (WHO) in 2003 to reflect the complexity of the endometrial gland and the presence or absence of cytological atypia. Accordingly, hyperplasias can be defined as simple or complex, with or without atypia. This system is designed to allow an estimate of the degree to which hyperplasia has progressed into endometrial cancer [4].

In addition to certain known factors, it has been shown in recent years that especially chronic inflammation has an especially significant role in the cancer's etiopathogenesis. It is known that during chronic inflammation various proinflammatory genes increase the production of many different mediators, resulting in apoptosis suppression, proliferation, angiogenesis, invasion, and metastasis [5]. The inflammatory response is a complex process, but activation of the nuclear factor-kappa beta (NF-kB) cascade is thought to be a central mechanism by which it is mediated [6].

NF-kB consists of multiple subunits with a common Rel homology domain. Inactive NF-kB is sequestered in the cytoplasm as it binds to its inhibitors (IKBs) until activation by IKB kinases as a consequence of oxidative stress and cellular damage. This results in the release and ubiquitination of IKB and the subsequent translocation of NF-kB dimers into the nucleus, regulating transcription of several genes that are mainly responsible for the local and systemic inflammatory responses [7].

NF-kB is known to play a role in chronic inflammation and cancer etiopathogenesis in endometrial adenocarcinoma, hyperplasia, and normal endometrial tissue. The present study is unique in the current literature in that the authors aim to use the immunohistochemical method to shed light on the role of NF-kB in the etiopathogenesis of endometrial cancer.

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Materials and Methods

Selection of patients

Patients were categorized into three groups based on their endometrial pathologies. The first group comprised patients with active bleeding complaints and benign pathologies, such as proliferative endometrium and endometrial polyps based on endometrial sampling. The second group comprised patients with endometrial hyperplasia based on endometrium biopsy and the third group consisted of patients diagnosed with endometrial cancer.

Pathological diagnosis of endometrial tissues

The cell sheet of patients with benign endometrial lesions who were diagnosed with endometrial hyperplasia and endometrial adenocarcinoma were retrieved from the pathology laboratory and re-examined. Simple endometrial hyperplasia without atypia was diagnosed in morphologies where the stroma-to-gland ratio increased in favor of gland; occasional encystations existed, but epithelial atypia was not present. Complex endometrial hyperplasia was diagnosed in morphologies where glandular congestion was more apparent and glandular architecture was complex in appearance.

The criteria for endometrial carcinoma diagnosis were the presence of stromal desmoplasia and cytological apparent atypia, with concomitant complex architecture with papillary and/or villous structures and cribriform formation. The archive glass sheets were reclassified according to the morphological diagnosis criteria described, and suitable block selection was made for immunohistochemical (NF-kB) staining.

Endometrial samples for immunohistochemical labeling of NF kappa B/65 (RelA) Ab-1

There are five different members of the NF-kB subunit family: p50, p65 (Rel A), c-Rel, RelB, and p52. These proteins form homo- and heterodimers, with the classic NF-kB heterodimer containing p50 and p65. Of these members of the NF-kB family, the p65 subunit is able to activate the transcription of target genes. Therefore, in the current study a specific kit containing antibodies against the p65 subunit of NF-kB was used to detect the level of activated NF-kB in endometrial samples.

As samples for immunohistochemical assay, four-micrometer paraffin sections on poly-L-lysine-coated slides were dried in an oven for one hour at 60ºC. The sections were then dewaxed in xylene, rehydrated in ethanol, and incubated for ten minutes in 3% H2O2 to block endogenous peroxidase. After washing in phosphate buffer saline (PBS), the sections were incubated for eight minutes in Ultra V Block. The immunoreaction was performed for 60 minutes with ready-to-use NF KappaB/p65 (Rel A) Ab-1 antibody (anti NF-kB p65, polyclonal PA 138279). After washing in PBS, slides were incubated with horse radish peroxidase (HRP) kit. Finally, the preparations were developed in AEC (3-amino-9-ethylcarbazole) chromogen counterstained with hematoxylin and mounted with aqueous-mount. To evaluate immunohistochemical NF-kB expression in the endometrium, the H-score method introduced by Budwit-Novotny *et al.* [8] was used. This semiquantitative method consists of summing the percentages of positively stained cells and multiplying these by a weighted intensity of staining: H-score = $\sum$ Pi (i+1), where ''Pi'' is the percentage of stained cells in each intensity category (0–100%) and "i" is the intensity, indicating weak (i=1), moderate (i=2) or strong staining (i=3).

Statistical analysis

The SPSS 15.0 program was used for the statistical analyses. The values were expressed as a mean ± standard deviation (SD) whenever appropriate. Categorical variables were reported as a number and a percentage. Normally distributed variables were compared using a one-way Anova test, whereas the Kruskal-Wallis test was used to compare non-normally distributed variables between the groups. For multiple comparisons of groups, Tukey and Conover tests were used where appropriate.

Results

The present study was conducted over three separate groups. The first group included benign patients based on endometrial tissue sampling; the second group included the patients with endometrial hyperplasia, and the third included the patients diagnosed with endometrial cancer. The first group consisted of 21 patients and the mean age value was found as 48.81 ± 12.22. Eight patients had menorrhagia, three patients had menometrorrhagia, and six had postmenopausal bleeding. In addition, descensus uteri were detected in two patients and uterine myoma in two other patients. Probe curettage was performed on ten patients, total abdominal hysterectomy and bilateral salpingo-oophorectomy in five patients, total abdominal hysterectomy in three patients, vaginal hysterectomy in two patients, and laparoscopic hysterectomy in one patient. When the pathology results of the patients were examined, proliferative endometrium was detected in ten patients; endometrial polyp in six patients, secretory endometrium in three patients, and atrophic endometrium tissue was detected in two patients.

The second group consisted of nineteen patients and the mean age value was found as 53.42 ± 5.65. Nine patients suffered from bleeding in the postmenopausal period; eight patients had menorrhagia and two had menometrorrhagia. Abdominal hysterectomy and bilateral salpingo-oophorectomy were performed in 14 patients, laparoscopic hysterectomy in three patients, only abdominal hysterectomy in one patient, and probe curettage procedure in one patient. Simple hyperplasia without atypia was detected in 12 patients, complex hyperplasia with atypia in three patients, complex hyperplasia without atypia in two patients, and simple hyperplasia with atypia was detected in two patients.

The third group comprised 19 patients, all of whom were diagnosed with endometrial adenocarcinoma, and the mean age value was 59.21 ± 11.6. Thirteen patients had postmenopausal bleeding and six had menorrhagia. Total abdominal hysterectomy + bilateral salpingo-oophorectomy and pelvic lymphadenectomy were performed in 13 patients and para-aortic lymphadenectomy and omentectomy were additionally performed in six patients. The mean number of pelvic lymph node resected was found as 19.05 ± 8.44 and mean number of para-aortic lymph node was 17.0 ± 4.97. Based on the staging system revised by FIGO in 2009, 13 patients were considered as Stage Ia, three as Stage Ib, two as Stage IIIc1, and one patient as Stage IIIa.

The assessment of immunostaining for NF-kB was made by using the H-score method. In the calculation of an H-score, a summation of the percentage of area stained at each intensity level multiplied by the weighted intensity of staining is generated. The average H score of the first group comprising benign endometrial pathologies was found as 102.4 ± 85.9; the average value of the second group with endometrial hyperplasia as 143.6±122.4, and the H score

Table 1. — *H score values and statistical analysis of the groups in the study.*

Patient Groups	H score	*p*
Group 1 (benign endometrial pathologies)	102.4±85.9	
Group 2 (endometrial hyperplasia)	143.6±122.4	
Group 3 (endometrial adenocarcinoma)	276.8±61.8	<0.001

of the third group diagnosed with endometrial cancer was found as 276.8 ± 61.8. When the average H score values of each of the three groups were compared with one another, it was seen that average values of groups 1 and 2 were similar ($p = 0.349$); however, average H score value of group 3 was significantly higher than that of the groups 1 and 2 ($p < 0.001$) (Table 1).

When the images from the light microscope of the immunohistochemical results of this study were examined, it was seen that cytoplasmic and/nuclear NF-kB expression was more apparent in the group diagnosed with endometrial cancer compared to the other two groups (Figure 1).

Discussion

While endometrial cancer is a major health issue in today's world, it is a pathology whose etiology has been partly re-

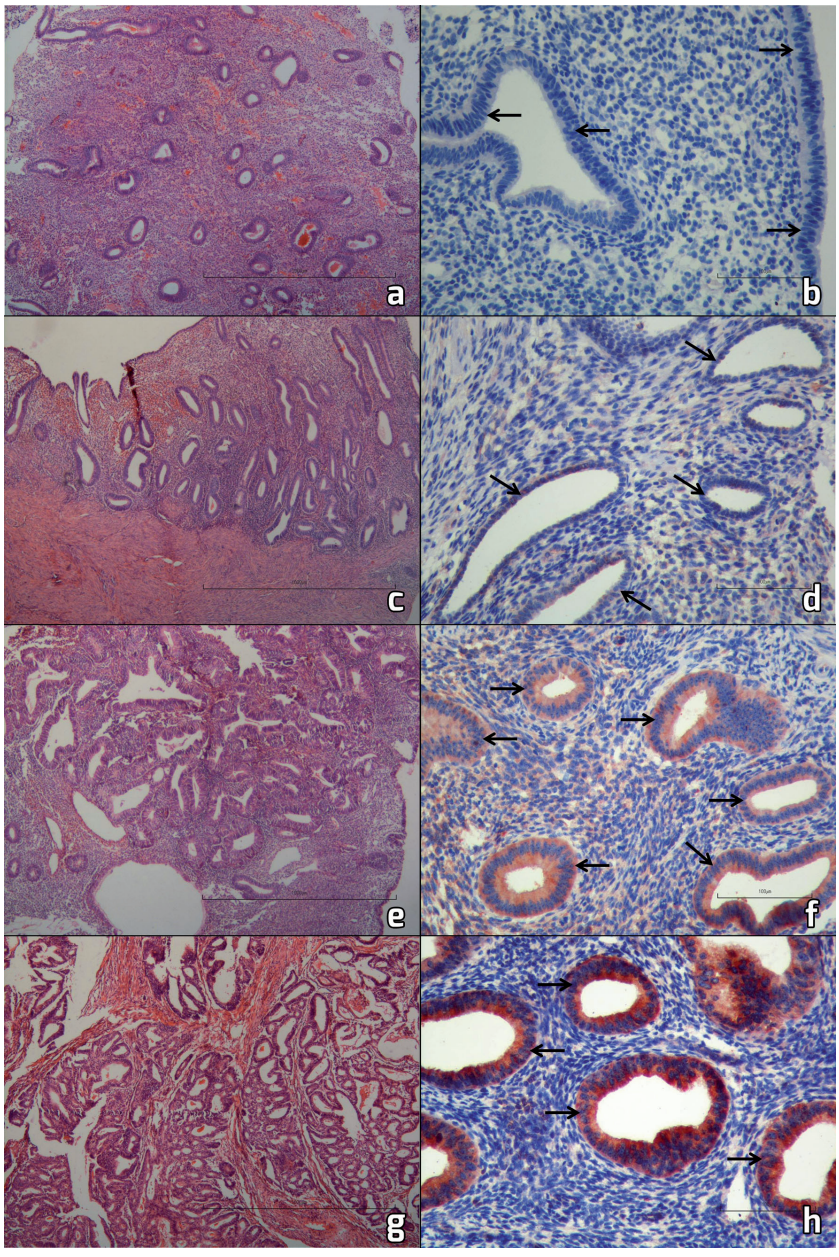

Figure 1: — a) Normal endometrial tissue, Hematoxylin Eosin (HE) stain. b) Normal endometrial tissue Nuclear Factor Kappa Beta (NFkB) immunohistochemistry method*. c) Simple hyperplasia without atypia HE stain. d) Simple hyperplasia NFkB without atypia immunohistochemistry method*. e) Complex hyperplasia with atypia HE stain. f) Complex hyperplasia NFkB with atypia immunohistochemistry method*. g) Endometrial cancer HE stain. h) Endometrial cancer NFkB immunohistochemistry method*. *Arrows show the NFkB positivity in immunohistochemical terms.

vealed. Hyperestrogenism, the best-known cause of endometrial hyperplasia and endometrial cancer, forces continuous mitosis in endometrium cells, causing somatic mutations by increasing DNA replication and associated DNA damage. Clinically, it occurs in situations such as early menarche, late menopause, anovulation, and polycystic ovary syndrome. Due to the fact that the menstrual cycle has an inflammatory effect on endometrial tissue, it can be concluded that the presence of unopposed estrogen results in hyperplasia of the endometrial glands and endometrial cancer through inflammation markers [9].

The connection between immunity and tumorigenesis was established in the 19th century, when Virchow detected structural changes and immune cells associated with chronic inflammation in the biopsy tissues of tumor cells [10]. Today, it is known that chronic inflammation plays a role in the etiology of numerous cancers, especially those of epithelial origin [11]. We now know that chronic inflammation contributes to 25% of all cancers and that as many as 15% of cancer-related deaths result from tumors associated with inflammation [12].

Increased levels of inflammatory mediators at the cell level induced by chronic inflammation cause tumorigenesis, angiogenesis, and cell progression. Increased levels of COX-2 cytokine (one of the most important mediators of chronic inflammation), and another component of the cyclooxygenase enzyme, COX-I (which ensures prostaglandin synthesis from arachidonic acid), have been seen in various premalignant and malignant lesions, such as colorectal cancer [13]. However, a reduction in cancer development as a result of anti-inflammatory medications that inhibit the synthesis of this cytokine have also been shown [14].

Chronic inflammation and inflammatory processes have also been identified as playing significant roles in the development of endometrial cancer. While increases in COX-2 production by immune cells secondary to inflammation and secretion of prostaglandin have been demonstrated in patients diagnosed with endometrial cancer, it has also been shown that use of anti-inflammatory agents reduces the strength of uncontrolled division in endometrial cancer cells and enhanced apoptosis in in-vitro media [15]. In addition to these cytokines, it has been proven that levels of mediators such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) interleukin-1 (IL-1) are increased in endometrial cancer cells [16].

The chronic inflammation that plays a major role in tumorigenesis, and the intracellular cytokines that form secondary to this are controlled by numerous regulator proteins in the cytoplasm and nucleus. The most commonly studied nuclear transcription in recent years is NF-kB [17], which controls cell proliferation, apoptosis, angiogenesis, immune reaction, cellular adhesion, and differentiation. The NF-kB pathway plays quite an important role in inflammation and tumor development [18]. The NF-Kb family consists of subunits such as p50, p65 (RelA), c-Rel, RelB, and p52. The subunit that is most common in human and other mammalian cells is p65/p50 heterodimer structure, which exists inside the cytoplasm connected to the inhibitor protein (IkB). Following internal gene mutation and/or external stimulation, IkB kinase enzyme is activated and the inactive NF-kB is translocated within the nucleus. This affects the transcription genes and stimulates local systemic inflammation and, indirectly, tumoral formation [19].

Many studies explain the relationship between NF-kB activation and cancer. Mosiaos *et al.* show that NF-kB activation caused by Ebstein-Barr virus efficiently brought about the etiopathogenesis of both Burkitt's and Hodgkin's lymphoma [20]. Similarly, significant NF-kB activation has been demonstrated at the cellular level in Hodgkin's lymphoma cases, spreading extranodally from hematopoietic cell tumors [21]. Another study by Nagel *et al.* indicated increased NF-kB activity in hairy cell leukemic cases [22]. The role of NF-kB activation has also been proven in the development of breast cancer, the most common cancer in women, in studies by Khan *et al.* [23] and others. A study by Lundqvist *et al.* showed that active vitamin D, which has become a popular product in recent years, achieves its antitumoral effect by inhibiting NF-kB [24]. Other studies show that NF-kB activation is effective in the etiopathogenesis of colorectal tumors. In a study by Moorchung *et al.* on 50 patients diagnosed with colorectal cancer, NF-kB expression was significantly increased in the experimental group by means of immunohistochemical analysis compared to the control group [25]. A similar study by Akca *et al.* showed increased NF-kB activation in lung cancer cells [26].

Other studies show NF-kB activation in the formation of gynecological tumors. A study conducted in malignant ovarian tumors proved that the MARCH7 molecule, which increases tumoral progression, uses the NF-kB pathway [27]. Furthermore, in their study on ovarian cancer, Lisanti *et al.* showed that NF-kB leads to tumoral cell proliferation by causing oxidative stress and cytokine release [28]. A similar study by Barlin *et al.* showed that NF-kB expression immunohistochemically increased in patients diagnosed with recurrent and primary serous ovarian cancer [29]. Of the many factors in the etiology of cervical cancer, HPV infection is the most significant. However, recent molecular studies suggest that NF-kB also plays a key role in the etiology of cervical cancer. The antitumoral effect of curcumin in the cell cultures of squamous cervical cancer patients was attributed to the substance's inhibition of the NF-kB pathway [30]. A review of the literature on NF-kB and endometrial cancer shows the present study to be unique in terms of materials and methods. Increased expression was observed in endometrial cancer cells in the in vitro cell culture in a study investigating NF-kB expression by means of the Western blot technique [31]. In another study, it was shown that bcl-2 and bax protein levels that had apoptosis protein decreased in tissues diagnosed with endometrial cancer. In the same study, NF-kB expression was immunohistochemically examined in cancer cells. Unlike the

present study, reduced expression was detected in the cancer tissue compared to the control group [32].

In conclusion, chronic inflammation and the resulting release of various cytokines by immune cells play a major role in the cancer etiology. These cytokines inhibit apoptosis and increase tumoral cell proliferation and angiogenesis. Numerous recent studies reveal that the factors resulting in chronic inflammation derive their effect from causing intracellular NF-kB activation. Increased NF-kB expression plays an important role in tumorigenesis in many systems, including gynecological tumors. The present authors believe that this study provides a major contribution to the literature, as it is the first, and so far the only study, to demonstrate NF-kB expression in the etiology of cancer using the immunohistochemical method.

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