MEDICAL SCIENCE MONITOR

CLINICAL RESEARCH

e-ISSN 1643–3750
© Med Sci Monit, 2020; 26: e922137
DOI: 10.12659/MSM.922137

Received: 2019.12.14
Accepted: 2020.02.11
Available online: 2020.03.23
Published: 2020.05.12

Gamma-Synuclein Levels Are Elevated in Peritoneal Fluid of Patients with Endometriosis

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Source of support: Departmental sources

Background: The role of gamma-synuclein (SNCG) has been widely examined in malignant conditions due to its possible role in disease progression, but very little information is available on its theoretical function on endometriosis formation.

Material/Methods: Between January 2016 and December 2016, we collected peritoneal fluid and plasma samples from 45 consecutive female patients, of which 15 were without endometriosis, 15 had minimal to mild endometriosis, and 15 had moderate to severe endometriosis. The statistical power was 0.98. We evaluated SNCG levels in the peritoneal fluid and plasma of patients diagnosed with endometriosis, and we compared them with the levels obtained from disease-free control subjects by using enzyme-linked immunosorbent assay.

Results: SNCG levels were statistically significantly (1.2-fold) higher in the peritoneal fluid of patients with endometriosis compared to controls (p=0.04). We did not find a significant difference between SNCG levels in the plasma of our endometriosis patients and the control group (p=0.086). However, despite previous data showing very limited expression of SNCG in healthy tissues, we found SNCG in the peritoneal fluid of all of the patients in our healthy control group.

Conclusions: Levels of SNCG were statistically significantly higher in the peritoneal fluid of patients with endometriosis compared to disease-free controls, which may indicate its possible role the formation and progression of the disease. Moreover, its biological function should be further investigated due to the conflicting results concerning its expression in healthy tissues.

MeSH Keywords: Endometriosis • Immunohistochemistry • Molecular Biology

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/922137

2232 2 2 26

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Background

Endometriosis is a chronic oestrogen-dependent disease that affects up to 15% of reproductive-age women [1]. In the presence of endometriotic lesions and subsequent adhesions, patients can experience pelvic pain and/or infertility [2]. Of the hypotheses aiming to explain the pathogenesis of endometriosis (e.g., coelomic metaplasia, Mullerian remnants, and retrograde menstruation) the most widely accepted is Sampson's theory of retrograde menstruation [3,4]. According to this, due to retrograde flow of mestruation, eutopic endometrium is shed to the peritoneal cavity. In patients who tend to develop endometriosis, this ectopic endometrium adheres to the peritoneal surface, forming the first appearance of the disease. There are multiple factors regulating the onset of endometrium adhaesion and invasion, such as aberrant expression of aromatase, citokines, and adhaesion molecules of the endometriosis implants and the peritoneal surface, resulting in adhesion and altered immune function in patients with endometriosis, interfering with humoral and cellular immune functions, and leading to inadequate removal of endometriosis implants [5,6].

Gamma-synuclein (SNCG), or breast cancer-specific protein-1, is a 13 kDa protein that is expressed in the peripheral and central nervous system, retina, and olfactory epithelium [7,8]. Its physiological functions are debatable, but it has been implicated in the pathogenesis of neurodegenerative diseases. Additionally, an elevation of the SNCG level has been shown in multiple types of gynecological tumors (e.g., ovarian, endometrial, and cervical) and non-gynecological tumors [9–13]. A comparison of normal breast and ovarian tissues with malignant tissues showed that SNCG is not expressed in healthy tissues apart from the ones listed above, but it is expressed in premalignant and malignant tissues [14]. In breast cancer, the demethylation of exon 1 of the SNCG gene has been shown to result in the aberrant expression of this protein [15].

Aberrant SNCG expression can lead to more aggressive phenotypes by inducing the expression and activity of matrix-metalloproteinases 2 and 9, leading to tissue adhesion and invasion [3,16]. The growth of a significant proportion of gynecological tumors, as well as benign diseases, is hormone-dependent, and SNCG can also interfere with oestrogen receptor alpha by stimulating its transcription [12]. Elevated SNCG levels have been shown in the endometrium of patients with endometriosis, as well as in endometriosis lesions, with higher concentrations in the latter [3]. Stronger SNCG staining has been found in the perivascular areas of the myometrium in endometriosis patients, suggesting its possible role in angiogenesis [3]. According to Edwards et al., endothelial cells of endometriosis implants and the perivascular area of the myometrium of subjects with endometriosis showed higher positivity by immunohistochemistry compared to the limited expression seen in eutopic endometrium. In their study, they did not differentiate adenomyosis cases among the group of enrolled subjects in the experiment. Using SP012, a peptide inhibitor of SNCG, led to reduced neovascularization in an animal model of endometriosis, suggesting the possible role of gamma-synuclein in the angiogenesis of endometriosis implants [3].

By regulating proliferation, adhesion, invasion, and oestrogen receptor signalling, we suspected that SNCG plays a role in disease formation and progression in endometriosis. Therefore, the aim of this study was to demonstrate differences in the peritoneal fluid and plasma SNCG levels of patients with endometriosis and healthy controls.

Material and Methods

Patients

Between January 2016 and December 2016, 45 patients awaiting laparoscopies were enrolled in our study. The patients were classified into 3 groups based on their intraoperative diagnoses. The control group consisted of women without endometriosis, and we divided the patients with endometriosis into 2 groups based on the intraoperative stages of the disease. In order to classify the severity of the disease, we used the revised American Fertility Society (rAFS) scoring system to divide the patients into minimal to mild endometriosis (rAFS I–II) and moderate to severe endometriosis (rAFS III–IV) groups [17]. The means of the ages of the control, minimal to mild endometriosis, and moderate to severe endometriosis groups were 31±9.5, 33±6, and 33±4, respectively. Those patients with fibroid(s) and/or hormonal treatments (oral contraception, dienogest, or gonadotropin-releasing hormone analog) were excluded from our study. For the protection of our human subjects, the study protocol was approved by the Institutional Ethical and Review Board of Semmelweis University in Budapest, Hungary. Informed consent was obtained from all of the patients before they were entered into this study (No: 143/2008).

Collection and preparation of samples

The peripheral blood samples were collected immediately before surgery, while the patient was on the operating table, before the induction of the anaesthetic drugs. The blood was collected in 4-ml Vacutainer K2EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) that were gently inverted several times immediately after drawing the blood to prevent formation of blood clots. The peripheral blood samples were centrifuged at 1811 g for 10 min at 4°C. Then, 500 µl of plasma from each sample was placed into a labelled Eppendorf tube (Axygen; Corning Incorporated, New York, NY, USA) and stored at –80°C until use.

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Table 1. Demographic and clinical data of the patients.

	Control	Stage I–II	Stage III–IV	p Value
Age (years, median)	33.5 (25–36)	33 (27–37.5)	32 (30–37)	0.919
BMI (kg/m^2, median)	22.3 (21.6–27)	21.8 (20–24)	22.3 (21–23.9)	0.482
Gravidity n (%)				
0 n (%)	8 (53)	10 (67)	10 (67)	0.68
1 or more n (%)	7 (47)	5 (33)	5 (33)	0.68
Previous surgery n (%)				
Laparoscopy n (%)	3 (20)	3 (20)	4 (27)	0.88
Laparotomy n (%)	3 (20)	2 (13)	2 (13)	0.84
Gynaecological symptoms				
Infertility n (%)	2 (13)	8 (53)	4 (27)	0.055
Abnormal uterine bleeding n (%)	4 (27)	1 (7)	0 (0)	0.054
Pelvic pain n (%)	7 (47)	11 (73)	14 (93)	0.018
Classification of endometriotic lesions				
No endometriosis n (%)	15 (100)	0 (0)	0 (0)	<0.0001
Superficial n (%)	0 (0)	12 (80)	15 (100)	
Endometrioma n (%)	0 (0)	1 (6.7)	11 (73.3)	
Deep infiltrating endometriosis n (%)	0 (0)	4 (26.7)	12 (80)	

BMI – body mass index.

To avoid blood contamination, after entering the pelvic cavity, the patient was put in the anti-Trendelenburg position before any additional operative manoeuvres were performed. All of the peritoneal fluid available was aspirated with a syringe. The peritoneal fluid sample was then centrifuged at 200 g for 10 min at room temperature. Afterwards, 500 µl of the peritoneal fluid supernatant was placed into a labelled Eppendorf tube and stored at –80°C until use. All of the specimens were stored in our biobank at Semmelweis University.

SNCG concentration measurements

The SNCG concentrations of the collected plasma and peritoneal fluid were measured using an enzyme-linked immunosorbent assay kit (SEA939Hu, Cloud-Clone Corp., Houston, TX, USA) according to the manufacturer's recommendations. The determination range was 31.25 pg/ml to 4000 pg/ml. Our results were at the lower end of the concentration range; therefore, we used the calibration equation to calculate the concentration values from the photometric results.

Statistical analysis

Using the Shapiro-Wilk's test, we found normal data distribution for the SNCG levels in the peritoneal fluid, but non-normal data distribution for the plasma. The Mann-Whitney U test was used to compare the SNCG levels. All of the tests were two-sided, and a p value<0.05 was considered to be statistically significant. Statistica, version 8.0 (StatSoft, Inc., Tulsa, OK, USA), was used for the statistical analysis.

Results

Patients and controls

The indications for surgery varied among the 45 patients, including infertility, pelvic pain, primary amenorrhea, and abnormal uterine bleeding. All of our patients were post-menarche and pre-menopausal. The statistical power was 0.98. The age, BMI, and anamnestic data of patients were comparable between the 3 groups. For detailed data regarding the subjects' anamnesis and the endometriosis patients' symptoms and disease phenotypes, see Table 1.

Peritoneal fluid SNCG levels

We found statistically significantly higher SNCG concentrations in the peritoneal fluid of the endometriosis patients when compared to the healthy controls (p=0.04) (Figure 1). SNCG levels were 1.2-fold higher in the peritoneal fluid of patients with endometriosis compared to disease-free controls. Moreover,

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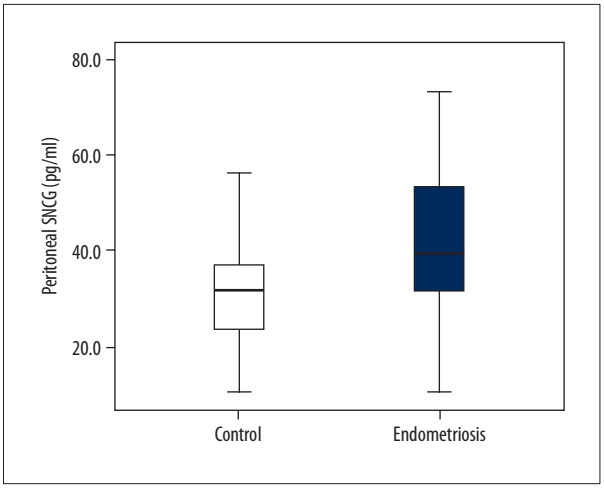

Figure 1. Gamma-synuclein (SNCG) levels in the peritoneal fluid of disease-free controls and endometriosis patients (p=0.04).

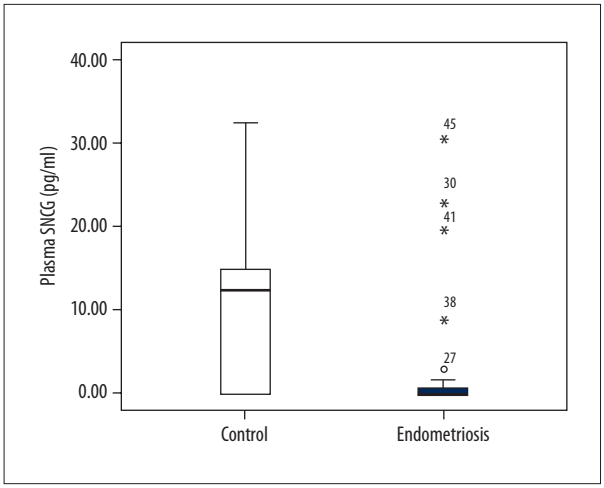

Figure 2. Gamma-synuclein (SNCG) levels in the plasma of disease-free controls and endometriosis patients (p=0.086).

Table 2. Revised American Fertility Society (rAFS) scores and gamma synuclein (SNCG) levels of the disease-free patients and the endometriosis patients, and the SNCG levels of the different endometriosis phenotypes.

	Control	All endometriosis cases	Stage I–II	Stage III–IV	Superficial +OMA	DIE	p Value
rAFS (median)	0 (0–0)	10 (7–41)	7 (4–8)	41.5 (26–61)	7 (4–10)	36.5 (17–61)	p*=0.00018 p**=0.1031 p#<0.00001
SNCG in peritoneal fluid (pg/ml)	31.5 (26–36.9)	39 (31–53)	36.9 (30.4–53)	39 (31.5–53.5)	43.8 (31.7–55.4)	34.3 (31–45)	p*=0.04 p**=0.66 p#=0.94
SNCG in serum (pg/ml)	10.7 (21.6–27)	0 (0–0.4)	0 (0–0.7)	0 (0–0)	0 (0–0)	0 (0–8.8)	p*=0.086 p**=0.97 p#=0.46

p* – p value between the controls and all of the endometriosis patients; p** – p value between the stage I–II and stage III–IV endometriosis patients; p# – p value between the superficial+ovarian endometrioma (OMA) and deep infiltrating endometriosis (DIE) cases.

SNCG was present in the peritoneal fluid samples of all 45 study subjects (100%). However, we found no significant difference between the SNCG levels of the patients with minimal to mild endometriosis and those with moderate to severe endometriosis. Additionally, there was no difference between the SNCG levels of the different endometriosis phenotypes (peritoneal endometriosis or ovarian endometrioma versus deep infiltrating endometriosis). The SNCG levels the peritoneal fluid samples of the study groups can be seen in Table 2.

Plasma SNCG levels

In the plasma samples, SNCG was detected in 6 patients (40%) in the control group, 4 patients (26.7%) in the minimal to mild endometriosis group, and in 2 patients (13.5%) in the moderate to severe endometriosis group. There was no statistically significant difference between the SNCG levels of the control group and endometriosis groups (p=0.086) (Figure 2). The plasma SNCG levels of the study groups are shown in Table 2.

In our group of 30 patients with endometriosis, visual analog scale (VAS) scores did not show any correlation with levels of SNCG in plasma or in peritoneal fluid. The surgeries were performed during the follicular phase in 63% (19/30) and in the luteal phase in 37% (11/30) of our patients. We found no correlation between SNCG plasma/peritoneal fluid levels and menstrual phases of the patients with endometriosis.

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Discussion

Based on the results of this study, the SNCG levels in the peritoneal fluid of the patients with endometriosis were statistically significantly higher when compared to those of the healthy controls. However, the SNCG levels in the plasma of all endometriosis patients were similar to the plasma levels of the healthy controls. The strength of our findings was that our data suggest wider implications for the role of SNCG in the biology of different gynecological diseases and, in particular, of endometriosis.

In several different endometrial carcinoma types (uterine papillary serous carcinoma and endometrial adenocarcinoma), elevated SNCG levels were detected using immunohistochemical, polymerase chain reaction (PCR) and Western blot testing methods. Additionally, via immunohistochemistry, elevated SNCG levels have been shown in aggressive ovarian cancer cases [18,20,22]. These data suggest a possible link between the pathogenesis of endometriosis and ovarian/endometrial cancer, as shown in previous publications [23,24].

To date, no data are available on the hypothetical SNCG level changes in the peritoneal fluid or plasma of patients with different gynecological tumors, but examinations on a histological level confirmed that elevated SNCG expression is associated with a more aggressive disease [10,12,19–22]. With regard to the SNCG levels in endometriosis, our data agree with the previous findings of Edwards et al. and Singh et al., who showed that the SNCG expression was elevated in the endometriosis lesions when compared to the eutopic endometrium in an animal model of endometriosis and in human endometrioma histological samples [3,16]. Both the results of Edwards et al. and Singh et al. are based on a histological semiquantitative analysis using immunohistochemistry [3,16]. In the present study, our aim was to perform quantitative measurement of SNCG levels in peritoneal fluid and plasma samples by ELISA. Our method allowed us to quantify secreted SNCG levels in both endometriosis-free subjects and in patients with endometriosis.

Our results showed statistically significantly higher SNCG positivity in the endometriosis patients' peritoneal fluid when compared to the controls, which agrees with previous transcriptional and histological SNCG data. Regarding the severity of endometriosis, we did not detect any significant differences between peritoneal SNCG levels of the 2 groups of patients with endometriosis (Stage I–II versus Stage III–IV). The role of SNCG has been shown mostly in the regulation of cellular proliferation, adhaesion, invasion, and neoangiogenesis, which are initial biological processes of disease formation. [12,15,16]. In our results of peritoneal fluid measurements SNCG levels were statistically significantly higher in patients with endometriosis compared to disease-free controls. Our results suggest that SNCG plays an more important role in disease formation than in disease progression, which might explain why we did not find any significant differences in patients with different severities of endometriosis.

In contrast, there was no difference between the plasma SNCG levels in the patients and controls. The fact that the SNCG levels were significantly higher in the peritoneal fluid of the endometriosis patients in our study suggests that SNCG plays a role in disease formation and progression via multiple pathways, such as angiogenesis, cellular proliferation regulation, and key matrix metalloproteinase expression locally (as seen in cases of pro-inflammatory oxylipins and cytokines), without plasma level changes [3,16,25,26].

To the best of our knowledge, we are the first to report that SNCG is secreted in the pelvis of endometriosis-free patients (without any benign or malignant gynecological conditions). According to our results, SNCG was secreted to the peritoneal fluid at a lower, but measurable level in all endometriosis-free controls and patients with endometriosis. However, considering plasma examinations, we found no significant difference in SNCG secretion in control patients when compared to the endometriosis group. Overall, our results showed that SNCG is secreted independently of endometriosis, and that it is present in the peritoneal fluid of healthy controls as well.

Bruening et al. reported that the expressions of alpha, beta, and gamma-synuclein were not detectable in normal ovarian tissue. In contrast, our results showed that SNCG was present in the peritoneal fluid of patients without endometriosis, which suggests that it is not only secreted in the disease-free nervous system, retina, and olfactory epithelium, but might also have a physiological function in other healthy tissues. We believe that the difference between our results and those reported by Bruening and coworkers is based on the fundamental difference between the methodology used in our experiments. Since the source of SNCG in the peritoneal fluid is unkown, our results of ELISA measurements of peritoneal fluid SNCG are not comparable with the histoligical, immunohistrochemistric analysis of ovarian samples. However, the physiological role of gamma-synuclein requires further elucidation [7,14].

Further research with larger sample sizes is needed to clarify the role of SNCG in healthy tissues and its possible effect on the pathogenesis of endometriosis.

Conclusions

Levels of SNCG are significantly higher in the peritoneal fluid of patients with endometriosis compared to disease-free

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controls, which suggests its possible role the formation and progression of the disease. Moreover, its biological function should be further investigated due to the conflicting results concerning its expression in healthy tissues.

Acknowledgements

The authors thank all of the women who participated in this study. The authors are also grateful to Nándor Ács, head of Department of Obstetrics and Gynecology at Semmelweis University, Budapest.

Conflict of interest

None.

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