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RESEARCH ARTICLE

Changes in Expression Pattern of SEMA3F Depending on Endometrial Cancer Grade - Pilot Study

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Abstract: ***Background*:** In the course of neoplastic diseases, a reduction in SEMA3F expression is observed, which translates into an increase in the proliferative and proangiogenic potential of cells forming the tumor and the surrounding microenvironment.

***Objective*:** The aim of this study was to determine the changes in SEMA3F level in endometrial cancer depending on its grade.

***Methods*:** The study material consisted of tissue samples: 15 without neoplastic changes (control group) and 45 with endometrial cancer (G1, 17; G2, 15; G3, 13; study group). SEMA3F expression was assessed using the immune-histochemical method.

***Results*:** The expression of SEMA3F was observed in the control group (Me = 159.38) and in the study group (G1, Me = 121.32; G2, Me = 0; G3, Me = 130.37). Differences between each grade and control and between individual grades were statistically significant. There were no significant correlations between SEMA3F expression and weight and Body Mass Index (BMI). The reduced SEMA3F expression in tumor tissue compared to healthy tissue indicates that this protein plays key roles in proliferation and angiogenesis.

***Conclusion*:** We found that depending on the severity of the disease, cancer adopts different survival strategies, where SEMA3F plays an important role. As a molecular marker, SEMA3F is not sensitive to weight and BMI.

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1. INTRODUCTION

Semaphorins (SEMAs) form a conservative family of proteins. Seven subclasses can be identified based on the structure, including transmembrane proteins (classes 1, 4, 5 and 6), secretory proteins (classes 2 and 3) and glycosylphosphatidylinositol-anchored proteins (class 7). Semaphorin 3F (SEMA3F) belongs to class 3 semaphorins [1, 2] and was initially characterized as a suppressor gene located on chromosome 3p21.3 and relatively often deleted during tumor development [3-6]. Further studies allowed to more accurately determine the role of SEMA3F in tumorigenesis [7]. Silencing of SEMA3F expression during neoplastic transformation was observed also with the participation of epigenetic mechanisms [8-11]. Antiproliferative and antiangiogenic activity of SEMA3F is the result of its interaction with neuropilin 1 (NRP1) and neuropilin 2 (NRP2) [7, 8, 12, 13]. The role of SEMA3F has been well studied and documented in breast

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cancer [14] and colorectal cancer [10, 15]. Although SEMA3F is known to have an effect on signaling pathways that are essential for carcinogenesis [10, 16], it is not sufficiently known whether SEMA3F affects signaling pathways in vascular endothelial cells [17]. SEMA3A, SEMA3B, SEMA3F have been shown to complement and enhance each other's actions, resulting in a reduction in tumor mass and suppression of tumor angiogenesis [18, 19]. It is also emphasized that the decrease in SEMA3A, SEMA3B, SEMA3F activity is an unfavorable prognostic marker of patient survival [20]. Therefore, taking into account the role of SEMA3F in neoplastic transformation [7] and the fact that molecular changes precede phenotypic changes, molecular diagnostics is gaining importance. Thorough understanding of molecular changes associated with carcinogenesis is an extremely important issue in the context of modern diagnostics and therapy of oncological diseases [21, 22].

The aim of this pilot study was to determine changes in SEMA3F level depending on the degree of endometrial cancer differentiation (G1-G3) compared to control and whether the assessment of its expression may be a complementary molecular prognostic marker in endometrial cancer. The relationship between SEMA3F expression at the mRNA (retrospective study) and protein levels was also analyzed.

2. MATERIALS AND METHODS

The study material consisted of tissue samples collected from patients who underwent hysterectomy: 15 without neoplastic changes (control group) and 45 with endometrioid endometrial cancer (study group). The study group was further divided according to the degree of histological differentiation [23]: G1, 17; G2, 15; and G3, 13. The exclusion criteria from the study group included extreme obesity (BMI>40), non-endometrioid endometrial cancer, adenocarcinoma with squamous elements, coexisting cervical carcinoma, endometriosis or adenomyosis, the use of hormone therapy 24 months prior to surgery. The analyzed groups of patients were characterized by the following anthropometric measurements: age, height, body mass (BM), body mass index (BMI), presented as mean ± standard deviation (C, 54.57 ± 10.81 years, 1.62 m ± 0.07 m, 73.79kg ± 11.95kg, 28.21 ± 8.20 - overweight; G1, 66.63 ± 7.05 years, 1.61 m ± 0.04 m, 74.38kg ± 11.95kg, 28.77 ± 5.73 - overweight; G2, 67.4 ± 11.89 years, 1.57 m ± 0.05 m, 84.77kg ± 25.63kg, 34.63 ± 11.63 - class I obesity; G3, 63.38 ± 9.52 years, 1.58 m ± 0.05 m, 83.13 kg ± 14.92 kg, 33.58 ± 6.31 - class I obesity). The study was approved by the Bioethical Committee of the Medical University of Silesia, no. KNW/0022/ KB/237/16. Informed consent was obtained from all of the patients recruited.

Expression profile of SEMA3F protein was determined in slides prepared from paraffin blocks provided by the Laboratory of Pathomorphology of Beskid Center of Oncology in Bielsko-Biała. Three slides were made (every fifth cut section) from one paraffin block. IHC staining was performed using rabbit anti-SEMA3F polyclonal antibody (Novus Biological). The prepared slides were incubated in citrate buffer (pH 6, 30 min at 95°C) in a water bath for antigen retrieval. They were treated with a solution of hydrogen peroxide (0.3% H2O2, 0.1% NaN3 in PBS) for 10 minutes to block endogenous peroxidase activity. In order to block any non-specific antibody binding, the slides were incubated for 30 minutes at room temperature in 1% BSA solution in PBS. Then, anti-SEMA3F was applied to the sections and incubated in a humidified chamber (20 h at 4°C). The avidin-biotin complex technique was used according to the manufacturer's instructions (Vectastain Elite ABC Kit, Vector Laboratories). The slides were stained with Gill's hematoxylin, dehydrated and coverslipped. Negative control was performed by replacing the primary antibody with rabbit IgG.

Photographic documentation was prepared using Nikon Eclipse E200 light microscope with Nikon DS-Fi1 digital camera. 15 photos were obtained for each patient (200x magnification). Images of necrosis or fibrosis were excluded. The intensity of staining was measured as the optical density of the reaction product in each positively stained cell. The average optical density was calculated with the use of image analysis program NISAR (Nikon). The statistical analysis was carried out using Statistica 12 PL software (StatSoft, Tulsa, Oklahoma, USA). The Shapiro-Wilk test was performed to assess the normality of the distribution of the obtained data. Kruskal-Wallis test was conducted to evaluate the statistical significance of the differences ($p<0.05$) followed by the multiple comparisons of mean ranks. Results are presented as median, lower and upper quartile. The correlations between SEMA3F expression and weight and BMI were also examined.

3. RESULTS

Semaphorin 3F was well expressed in all control samples. The reaction to this protein was only visible in glandular cells. In all examined grades of endometrial cancer, SEMA3F expression was revealed only in cancer cells. In G1 endometrial cancer, SEMA3F was well expressed in approximately 70% of the analyzed samples, achieving 76% of the control. In G2 endometrial cancer samples, SEMA3F expression was below the sensitivity level of the assay. In turn, SEMA3F was well expressed in 65% of G3 endometrial cancer samples, reaching 82% of the control (Fig. **1**, Table **1**). There were no significant correlations between SEMA3F expression and weight and BMI.

4. DISCUSSION

In our previous study, we evaluated the changes in the transcriptional activity of genes involved in angiogenesis in women diagnosed with endometrial cancer (G1-G3). It was indicated that as the cells forming the tumor de-differentiated, the expression of SEMA3F increased (G1, FC = -1.2025467; G2, FC = -1.034659; G3, FC = 1.5599537) [24]. These results became the first premise to assess the changes in SEMA3F at the protein level, based on the immunohistochemical reaction. In addition, it should be taken into account that it is extremely important to broaden the knowledge about molecular mechanisms related to cancer, which allows the development of new diagnostic and therapeutic algorithms [21].

An important factor determining the analysis of SEMA3F expression in the context of angiogenesis and cancer is the fact that the signaling pathways activated by the SEMA3F

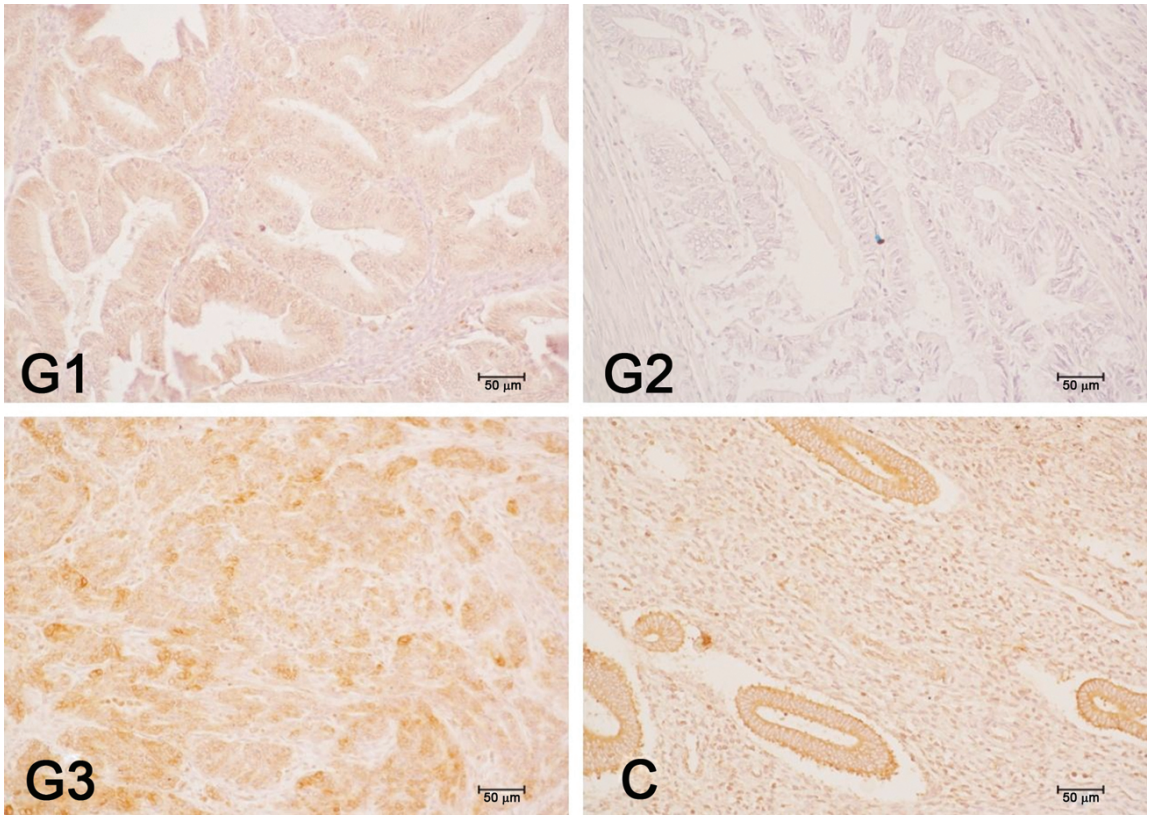

Fig. (1). Immunohistochemical localization of SEMA3F in different grades of endometrial cancer. C, control; G, a grade of endometrial cancer. 200x magnification.

Table 1. Optical density of the reaction product of SEMA3F in different grades of endometrial cancer and control.

Localization	-	C	G1	G2	G3	Kruskal-Wallis Test	Post-hoc Test
Glandular cells	Me	159.38	-	-	-	0.000	G1 *vs*. C p=0.000000 G2 *vs*. C p=0.00 G3 *vs*. C p=0.000000 G2 *vs*. G1 p=0.00 G3 *vs*. G2 p=0.00 G3 *vs*. G1 p=0.045169
	Q1	146.54	-	-	-		
	Q3	172.63	-	-	-		
Cancer cells	Me	-	121.32	0	130.37		
	Q1	-	110.45	0	118.38		
	Q3	-	132.28	0	140.32		

are still not sufficiently known in the vascular endothelium [17, 25], although it is indicated that SEMA3F may act as a marker of angiogenesis and lymphangiogenesis [26]. It is also indicated that SEMA3F acts as an inhibitor of the PI3K-Akt-mTOR signaling pathway in T cells, cancer cells and endothelial cells. In addition, the importance of SEMA3F in the formation of the tumor microenvironment is underlined [12]. It is also noted that SEMA3F is not only characterized by antiangiogenic properties, but it can also inhibit the growth of cells that have undergone neoplastic transformation [27-29]. As part of this preliminary study, the level of SEMA3F was assessed depending on the degree of endometrial cancer differentiation. The following dependence of SEMA3F expression can be observed: C>G3>G1>G2. The comparison of the results obtained in the microarray and immunohistochemical analysis seems to confirm the thesis about the epigenetic regulation of SEMA3F expression [8-11, 30]. However, regardless of the cancer grade, the SEMA3F expression is always lower than in the control. These observations seem to confirm the role of SEMA3F and other researchers' assessments of the expression profile of this protein in cancer tissues compared to normal tissues [5, 6, 12]. In G2 endometrial cancer, SEMA3F expression was silenced compared to G1 or G3 cancer. If the expression at all degrees of differentiation was below the sensitivity level of the method, it could be assumed that the reason is SEMA3F deletion, which would explain the proliferative and proangiogenic properties of cells forming the tumor and blood vessels

that supply it with oxygen and nutrients. However, in this pilot study, the mentioned expression pattern was not observed. Such a sudden drop, followed by an increase in the SEMA3F level, may result from the regulatory role of epigenetic factors in establishing gene expression. Our results may suggest that DNA methylation is the most probable mechanism affecting the observed expression profile. This seems likely because the attachment of the methyl group most often to the cytosine of the CpG island is a reversible modification [31]. Thus, it can be assumed that in G2 endometrial cancer methylation of the SEMA3F promoter regions is observed, whereas in G3 the methylation pattern is erased. Therefore, the most intense cell divisions, the ability to metastasize and the formation of new blood vessel networks occur in G2 endometrial cancer, where the cells are deprived of the suppressor effect of semaphorin 3F [3].

Interesting observations were made by Richeri *et al.* In studies on rat uterus, it was found that estrogens strengthen the expression of *SEMA3F*. The important role of *SEMA3F* and its function in angiogenesis and disorders of the peripheral nervous system of patients with endometriosis was indicated [32]. Observations on the regulatory effect of estrogens on SEMA3F expression are confirmed by Edjekouane et al [33]. In this pilot study, when analyzing the changes in SEMA3F expression between tissue samples, there was a higher level of protein in G3 endometrial cancer compared to G2 and G1. One of the reasons (other than epigenetic) of these differences in expression may be the increasing concentration of estrogens as the tumor-forming cells lose their homogenous character. Thus, the number of estrogen receptors, through which specific biological effects are exerted, would increase [34]. The second reason may be the fact that the cancer development strategy changes in grade 3. It seems that the activity of cells that form the tumor and its microenvironment, including the blood vessels, is directed to ensure an adequate supply of oxygen and nutrients only to the cells of the primary tumor mass [23, 24]. Therefore, an increase in the expression of SEMA3F in G3 may indicate a reduction in the tumor's ability to metastasize and the formation of new blood vessel networks [17, 19]. Moreover, it should be remembered that the changes in the SEMA3F level observed in this work may be the result of de-differentiation of endometrial cells that lose their surface markers. This probably contributes to changes in cellular signaling, its dysregulation and the progressive loss of homeostasis in the environment. Ferreira *et al.* analyzed changes in SEMA3A-3F expression in the proliferative and secretory phase of the menstrual cycle. They observed a different expression profile of *SEMA3F* at the transcriptome level compared to other *SEMA3* representatives. They found that the observed expression pattern of *SEMA3F* indicates that it does not participate in the proliferation of endometrial cells and that the protein is primarily expressed in the secretory phase of the menstrual cycle [35]. They also agree with the critical role of SEMA3F in cancer development as a factor counteracting tumor angiogenesis [36]. However, other research teams do not support the conclusion that SEMA3F expression is determined by the cycle phase. The following discrepancies may be the result of different inclusion criteria for the study and control groups, the size of groups, research methodology [37]. Gao *et al.* found a decrease in SEMA3F expression in colorectal cancer samples compared to the non-tumor colorectal tissues. Moreover, using qMSP, they verified that during neoplastic transformation, the SEMA3F promoter regions undergo methylation, which results in the silencing of SEMA3F expression. Their assumptions regarding the role of this epigenetic mechanism in the regulation of the discussed semaphorin were confirmed by exposing CRC cell lines to 5-Aza-2'-deoxycytidine (5-Aza-CdR), which restored the methylation pattern observed in the control [38]. Thus, it appears that the increase in SEMA3F expression translates into anti-proliferative and anti-migratory effects as well as reduction of metastatic potential [15]. Nguyen *et al.* also observed that the progression of changes observed in endometrial cancer leads to the silencing of SEMA3F expression [39]. Observations consistent with our results are presented by Scheerer *et al.*, who analyzed the expression of SEMA3F in peritoneal fluid of 14 women with endometriosis (137.7 ± 26.02 ng/ml) compared to healthy women (201.7 ± 24.82 ng/ml). This indicates that the inflammation accompanying, among other things, endometriosis, reduces the level of SEMA3F, and thus increases the angiogenesis and proliferation of cancer cells [37, 40]. However, Scheerer *et al.* did not notice any differences in the SEMA3F expression in women with endometriosis who experienced pelvic pain compared to those who did not [37]. The strengths of our pilot study are strict inclusion and exclusion criteria, as well as a comparison of the obtained data with the results from our previous report. On the other hand, it would be preferable to increase the size of the study and control groups in accordance with the current inclusion and exclusion criteria. In addition, it would also be valuable to confirm the obtained results by other methods.

In our preliminary report, we did not find a statistically significant correlation between SEMA3F level and weight and BMI, which seems to increase its chances of becoming a complementary molecular marker, because it is not sensitive to these two parameters, which undergo frequent and relatively large changes during carcinogenesis [41]. It has been observed that silencing of SEMA3F expression intensifies neoplastic changes. It is possible that such action may depend on the type of tissue. This confirms the complexity of molecular mechanisms occurring during cancer, thus indicating the utilitarian aspect of molecular studies [24].

CONCLUSION

SEMA3F, referred to as tumor suppressor gene, is getting better characterized thanks to the development of molecular biology techniques. The observed changes in the SEMA3F level, depending on the degree of endometrial cancer differentiation (G1-G3), may indicate the participation of epigenetic mechanisms in the regulation of its expression. We observed high dynamics of SEMA3F expression changes between individual grades of endometrial cancer, which indicates that depending on the severity of neoplastic changes, the strategy of development and cancer cell survival changes. SEMA3F seems to be worth considering a new complementary molecular marker for endometrial cancer.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Approval of the Bioethical Committee of the Medical University of Silesia, no. KNW/0022/KB/237/16 has been obtained for this study.

HUMAN AND ANIMAL RIGHTS

No animals were used in this study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

CONSENT FOR PUBLICATION

Informed consent was obtained from all of the patients recruited.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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