**RESEARCH ARTICLE**

**Differences in the Expression Pattern of mRNA Protein SEMA3F in Endometrial Cancer *in vitro* under Cisplatin Treatment**

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**Abstract:** Background: Semaphorin 3F (SEMA3F) plays a substantial role in carcinogenesis, because of its role in inducing angiogenesis, and creating a microenvironment for the developing tumor.

**Objective:** The purpose of this work was to assess the impact of cisplatin, depending on the concentration and exposure time on the expression pattern of SEMA3F in an endometrial cancer cell line.

**Materials and Methods:** Cultures of the Ishikawa endometrial cancer cells were incubated with cisplatin with the following concentrations: 2.5µM; 5µM; and 10µM and for the following periods of time: 12; 24; and 48 hours. Cells not incubated with the drug constituted the control in the experiment. To determine the effect of cisplatin on the expression of SEMA3F, the real-time quantitative reverse transcription reaction (RtqPCR; mRNA) was used, as well as the ELISA assay (protein). The statistical analysis was done with the admission of p<0.05.

**Results:** The silencing of SEMA3F expression on the transcriptome and proteome levels in a culture unexposed to the effects of cisplatin (p<0.05) were noted. Along with an increase in the concentration of the drug used, the number of copies of the gene transcript, during the shortest incubation period had a gradual increase. Only for the highest concentration of the drug, substantial statistical differences in the expression of the SEMA3F protein between 24 and 48 hour incubation periods (p<0.05) were determined.

**Conclusion:** Using cisplatin in an endometrial cancer cell culture results in an increased expression of SEMA3F, which advantageously affects the normalization of the neoplastic angiogenic process and lowers the proliferation of the cells making up the mass of the tumor.

**Keywords:** SEMA3F, endometrial cancer cell line, cisplatin, expression, supplementary molecular marker, gene transcript.

1. **INTRODUCTION**

Semaphores (SEMA) are a family of proteins, which can be divided into seven subclasses found in vertebrates. Semaphores of classes 1,4,6 and 7 are transmembrane proteins, members of classes 2 and 3 are characterized as secretory proteins, during which members of class 7 are proteins anchored in glycoprophosphatidylinositol [1]. Semaphores show an ability to interact with neuropilin receptors or plexins, the result of which is the initiation of the mentioned signaling cascades [2, 3]. They have a significant role not only in processes but also mainly in organogenesis, modulating the immunological response, maintaining the rate at which new blood vessels are created, and conditioning the adhesion of cells to the foundation [1-3].

The incorrect expression of certain members of the semaphorin family was determined during the neoplastic transformation process and the process of creating new blood vessels (angiogenesis) and lymphatic vessels (lymphangiogenesis), which are directly tied but does not allow itself to be controlled [4, 5]. One of the members of the aforementioned family of proteins is SEMA3F, the silencing of its expression is observed in tumors, which constitutes a disadvantageous prognostic marker. It is pointed to that during the neoplastic transformation process, it leads to a deficiency of the aforementioned gene, which is located on chromosome 3p [6]. Therefore, also halting the secretion of SEMA3F results in excessive proliferation of cells in comparison to...
apoptosis, increasing the potential for metastasis by the modified tumor cell [7, 8].

In physiological conditions, SEMA3F is a strong angiogenic factor, and its effect is caused by competition with the Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor-Beta 1 (TGFβ1) for connecting with the neuropilin receptors and continued transduction of signal [9, 10]. The second mechanism through which SEMA3F halts the creation of new blood vessels is based upon promoting the apoptosis of endothelial cells [11, 12].

Analysis of the expression profile of SEMA3F not only in carcinogenesis but also under the influence of the used treatment has a key meaning for a better understanding of the molecular foundation of neoplastic changes and monitoring the effectiveness of therapy [13, 14].

One of the most common gynecological tumors diagnosed is endometrial cancer [15], which in advanced forms is treated with cisplatin [16], the mechanism of which is connected with the induction of damaged DNA, the endoplasmic reticulum as well as mitochondria. In the molecular aspect, the activity of cisplatin strongly influences the pathways connected with apoptosis and oxidative stress [17].

One of the useful models of in vitro endometrial cancer used to evaluate changes on the molecular level is the Ishikawa cell line, it was obtained from a well-varied human endometrial adenocarcinoma.

The purpose of this work was to assess the impact of cisplatin on the expression profile of SEMA3F in Ishikawa endometrial cancer cells. Moreover, this evaluation may be helpful to indicate the usefulness of SEMA3F as a supplementary molecular marker in endometrial cancer diagnosis and therapies.

2. MATERIALS AND METHODS

2.1. Cell Culture

In this study, the Ishikawa endometrial cancer cell line was used as a material. These cells were exposed to cisplatin at concentrations of 2.5 µM, 5 µM and 10 µM for 12, 24 and 48 hours periods. The untreated cells constituted the control in this work. The cells were routinely grown in the Minimum Essential Medium (MEM) supplemented with 2 mM glutamine, 1% Non-Essential Amino Acids (NEAA), and 5% Fetal Bovine Serum (FBS) according to the manufacturer’s protocol. Cultures were incubated under conditions of a constant temperature of 37°C and a 5% CO₂ enriched atmosphere. Reagents used in this part of this study were bought from Sigma Aldrich, St Louis, MO, USA. Three replicates of each time and concentration were performed. After 24 hours, cisplatin was added to the culture of cells in six-well plates. The control culture was carried out separately for each time, i.e., for 12, 24 and 48 hours in three replicates.

2.2. XTT Assay

In the first step of this study, proliferation and cell viability of the Ishikawa endometrial cancer cell line under different concentrations of cisplatin and exposure time of the cells to the drug by XTT assay was evaluated (In Vitro Toxicology Assay Kit, XTT based; Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer’s protocol.

2.3. Invasion Assay

Invasion of Ishikawa endometrial cancer cells under cisplatin treatment was evaluated by using a 24-well Transwell chemotaxis chamber (Costar, USA) with an 8mm pore size membrane coated with BD Matrigel™ Matrix (BD Biosciences, USA). First, the control cells (cells unexposed to cisplatin) and the Ishikawa cultures treated with 2.5 µM, 5 µM, and 10 µM of cisplatin for 12, 24 and 48 hours were suspended in 200 ml serum-free DMEM medium, then these cells were filled in the upper chamber. Next, 600 ml of complete medium was added and incubated for 12 hours. Hema-toxylin was used to stain cells and they were microscopically counted.

2.4. Apoptosis Analysis

The Annexin V-FITC/PI apoptosis detection kit (Sigma Aldrich, Product no. APOAF-20TST) was used for analyzing the influence of cisplatin on cell apoptosis. The Annexin V-FITC kit allows for the fluorescent detection of annexin V that is bound to apoptotic cells and additionally quantitative determination through flow cytometry. The AnnexinV-FITC kit uses annexin V conjugated with Fluorescein Isothiocyanate (FITC) in order to label phosphatidyserine sites on the membrane surface. This kit includes Propidium Iodide (PI) for the labeling of cellular DNA in necrotic cells when their cell membrane has been completely compromised. This combination allows for differentiation between early apoptotic cells (annexin V positive, PI negative), necrotic cells (annexin V positive, PI positive), and viable cells (annexin V negative, PI negative).

2.5. Expression of SEMA3F and of TGFβ1 and VEGF (as SEMA3F-Related Genes) on mRNA and the Protein Level

To determine the changes in the expression of SEMA3F, and related genes, such as TGFβ1 and VEGF under cisplatin treatment, the real-time quantitative reverse transcription reaction (RTqPCR) and the ELISA assay, were performed.

In the RTqPCR assay, SensiFAST SYBR No-ROX One-Step Kit (Bioline, London, UK) was used, as recommended by the manufacturer. The thermal profile of this reaction was as follows: reverse transcription (45°C for 10 min), polymerase activation (95°C for 2 min), 40 cycles including denaturation (95°C for 5 s), annealing (60°C for 10 s), elongation (72°C for 5 s). As an endogenous control, β-actin (ACTB) was used.

The second stage of this experiment was associated with indicating the influence of cisplatin on the expression profile of SEMA3F on the protein level through the ELISA assay.

The SEMA3F (Cat.No MBS161003, MyBioSource, San Diego, CA 92195-3308, USA) kit was used. The assay was performed at room temperature after all necessary reagents, standard solutions and previously used samples were prepared. Standards containing the biotinylated antibody were added to each well at 50µl. In turn, 10 µl of the anti-
SEMA3F antibody was added to the wells with the introduced samples in the amount of 40 μl, followed by 50 μl of streptavidin-HRP solution. The entirety was incubated for 60 minutes at 37°C, after which the wells were washed 5 times with a wash buffer (60 seconds/wash). In the next step, 50 μl of substrate A solution per well was added, followed by the addition of 50 μl of substrate solution B per well and again incubated for 10 minutes at 37°C in the dark. After this time, 50 μl of stopping solution was added, which resulted in a change in the color of the well contents from blue to yellow. In the last step, the optical density was determined for each sample at 450 nm and the concentration of SEMA3F was determined depending on the dose of cisplatin and the time of incubation of the cells with the drug.

2.6. Analysis if Cisplatin Exerts its effect via SEMA3F Signaling Pathways

The last step of our study was associated with assessing if cisplatin exerts its effect via SEMA3F signaling pathways by using target-specific 19-23 nucleotide siRNA oligo duplexes designed to knock down SEMA3F expression (SEMA3F siRNA Human; Catalog# MBS8213103, MyBioSource, San Diego, CA, USA) and 21 nucleotide siRNA Negative Control (Catalog# MBS8241404, MyBioSource, San Diego, CA, USA). Both untreated and exposed to cisplatin endometrial cancer cells were transfected by SEMA3F siRNA according to instruction. In order to confirm the influence of cisplatin on SEMA3F-related pathways, the expression of SEMA3F via RTqPCR and the ELISA assay were assessed.

2.7. Statistical Analysis

The licensed version of Statistica 13.0 PL (StatSoft, Cracow, Poland) was used in the statistical analysis of data related to changes in the expression profile of SEMA3F.

As the Shapiro-Wilk test (p<0.05) confirmed the normality of the data distribution, the next steps of analysis were made by using parametric tests. The ANOVA variance test was also conducted, and the analysis indicated that the differences in the results were statistically significant and the posthoc Tukey test was also performed (p<0.05).

3. RESULTS

To assess the XTT assay results, the absorption value observed in the control cell (untreated endometrial cancer cells to cisplatin) cultures was assumed to be 100%. It was used as a comparison value for the cells exposed to the drug. The results showed that regardless of the concentration of cisplatin used in the experiment, the number of viable cells decreases as the incubation time increases. It can be observed that cisplatin in the concentration of 5 µM causes the death of about 50% of endometrial cells incubated with it (half maximal inhibitory concentration; IC50). Statistically significant differences were showed between the cells treated with cisplatin in comparison to the control cell culture (Fig. 1; p<0.05).

Results of the invasion assay showed that cisplatin in the range of concentrations used statistically significantly inhibited Ishikawa cell invasion when compared to a control culture (p<0.05; Fig. 2). The fastest progressive decline in invasive cells was observed in the culture exposed to 5 µM cisplatin (from 61.9% after 12 hrs by 42.6% after 24 hrs to 41.02% of invasive cells after 48 hour-long incubation time with the drug). The obtained results also showed that the percentage of non-invasive cells was increasing with drug concentration and incubation time with it (Fig. 2).

The next stage of our work was to evaluate endometrial cancer cell apoptosis induced by cisplatin. As shown in Fig. (3), cisplatin increased the percentage of apoptotic cells in a dose and in a time-dependent manner in endometrial cancer cells compared to the control culture (p<0.05).

In turn, Table 1 presents changes in the expression of the SEMA3F mRNA; the gene coded by this protein depending on the dose of cisplatin; time of exposure of the endometrial cancer cell to the effect of the drug.

First of all, it should be noted that not only on the transcriptome level but also on the proteome level, a decrease in

Fig. (1). The results of cytotoxicity and proliferation XTT assay in Ishikawa endometrial cancer cells under cisplatin treatment. (A higher resolution/colour version of this figure is available in the electronic copy of the article).
the expression of SEMA3F in the unexposed to cisplatin culture was observed, when compared to the endometrial cancer cells under the effect of the chemotherapeutic agent (p<0.05).

Concerning the matter of the concentrations of cisplatin used, it can be observed that alongside the elongated exposure time of the cells to the drug, the expression on the mRNA level increases successively, similarly to what happens on the protein level. Nonetheless, for the concentration of 5 µM, it can be determined that the exposition lasting an entire day causes a decrease in expression in comparison to the 12-hour incubation, wherein extending the amount of time the drug affects the endometrial cancer cells to 48 hours caused an increase in the transcriptional activity of SEMA3F.

Based on the obtained results, it can also be observed that with an increase in the concentration of the drug used, the number of the transcription gene copies increases, with the shortest amount of incubation time, it is subjected to a gradual increase.

Also, on the protein level, it can be determined that cisplatin induces changes on the SEMA3F level, no matter the length of time or dosage of the drug used (p<0.05). Statistical analysis indicates that there were statistically significant changes between the level of SEMA3F between certain incubation times, which are induced at the same concentration of cisplatin. However, only for the highest concentration of the drug, substantial statistical differences in the expression of SEMA3F between the times of 24 and 48 hours of incubation (p<0.05) were determined, for the other concentrations in this comparison, this wasn’t the case (p>0.05).

In turn, the last step of the molecular analysis was to make sure that cisplatin exerts an effect via SEMA3F dependent signal pathways. The results of the expression profile of mRNA TGFβ1 and VEGF under cisplatin treatment were presented in Table 2.
**Table 1.** The expression profile of SEMA3F depending on the concentration and the amount of exposure time of endometrial cancer cells when undergoing cisplatin treatment.

| Concentration of Cisplatin [µM] | Time [Hours] | RTqPCR (Copy Number of SEMA3F/1µg of total RNA) | RTqPCR (Copy Number of β-actin/1µg of total RNA) | ELISA Assay SEMA3F [ng/ml] |
|-------------------------------|--------------|-----------------------------------------------|-----------------------------------------------|-----------------------------|
|                               |              | Mean                               | Standard Deviation                          | Mean                             | Standard Deviation |
| 0                              | 12           | 2579974                             | 52669                                         | 72770                           | 1012              | 7.991             | 0.026             |
| 0                              | 24           | 2461323                             | 45863                                         | 64139                           | 694               | 7.715             | 0.058             |
| 0                              | 48           | 2349829                             | 71402                                         | 15083                           | 745               | 6.71              | 0.066             |
| 2.5                            | 12           | 3732235a                           | 335561                                        | 72403                           | 968               | 9.230             | 0.06              |
|                               | 24           | 2703216a,b                         | 54062                                         | 12655                           | 1002              | 10.688            | 0.019             |
|                               | 48           | 5144625a,c,d                       | 156403                                        | 61875                           | 1058              | 10.830            | 0.036             |
| 5                               | 12           | 9371474a                           | 1670029                                       | 132600                          | 786               | 10.960            | 0.053             |
|                               | 24           | 6967012a,b                         | 174888                                        | 150900                          | 30012             | 11.377            | 0.020             |
|                               | 48           | 8740369a,c,d                       | 265717                                        | 128500                          | 21000             | 11.487            | 0.021             |
| 10                              | 12           | 19974217a                         | 399464                                        | 77825                           | 985               | 11.524            | 0.007             |
|                               | 24           | 174913193a,c,d                    | 2701477                                       | 38083                           | 1047              | 11.523            | 0.006             |
|                               | 48           | 19319607a,c,d                     | 403948                                        | 180306                          | 698               | 12.103            | 0.012             |

- statistically significant differences in expression of SEMA3F between cells exposed to cisplatin vs control p<0.05.
- statistically significant differences in expression of SEMA3F between 12 vs 24 hours exposition of cisplatin p<0.05.
- statistically significant differences in expression of SEMA3F between 24 vs 48 hours exposition of cisplatin p<0.05.
- statistically significant differences in expression of SEMA3F between 12 vs 48 hours exposition of cisplatin p<0.05.

It can be observed that cisplatin changes the expression profile of SEMA3F-related genes on the transcriptome level. The analysis showed that TGFβ1 was upregulated, while VEGF was down regulated in the cell cultures exposed to cisplatin. The effect of the drug on the expression of selected genes was confirmed by more visible changes with a higher dose of cisplatin and a longer exposure time. Statistical analysis showed that differences in the expression pattern of mRNA TGFβ1 and VEGF between Ishikawa endometrial cancer cell lines incubated with cisplatin compared to a control culture constituting of untreated cells are statistically significant (p<0.05; Table 2).
Similarly, these authors also acknowledged that the expressiveness of therapy was smaller than the expected value.

SEMA3B were characteristic of patients in whom the effectiveness of therapy was smaller than the expected value. SEMA3F and SEMA3B were characteristic of patients in whom the expression of SEMA3F was lower than the expected value.

Beta 1 (TGF-β1) [21]. It is important to keep in mind the observations made by Beuten et al., which contrast with the generally accepted role of SEMA3F [6-12]. This could indicate that SEMA3F could be markers that suggest a negative prognosis of disease remission duration [18]. In turn, Li et al. indicated that in a group of 198 patients, the expression of SEMA3F in the group of patients with a low-and intermediate-risk of localized prostate cancer was not significantly different in the assessed groups and did not correlate with experience. The materials used for this study were biopsies obtained during a radical prostatectomy [19]. An interesting observation regarding the relationship between the expression of SEMA3F and the intensification of cancerous changes as well as regarding the answer to treatment were made by Drenberg et al., together with the intensification of the severity and advancement of changes in the duration of ovarian cancer they determined an ever lower expression of SEMA3F, and in some of the patients a complete silencing of expression. Simultaneously they indicated that only the severity of the tumor correlated with a decreased concentration of SEMA3F and advancement of the disease [20]. However, there is a lack of research regarding the effects of cisplatin on the expression of SEMA3F in the in vivo model.

Bearing in mind the cited results of research [18-20] together with the observations cited in our own study, it seems that the level of expression of SEMA3F is dependent not only on the aggressiveness of cancer but also on its location. It is important to keep in mind the observations made by Beuten et al., which contrast with the generally accepted role of SEMA3F [6-12]. This could indicate that SEMA3F could be an advantageous or disadvantageous marker of survival or response to treatment depending on the biological context, as is in the case of, for example, Transforming Growth Factor-Beta 1 (TGF-β1) [21].

Therefore, the carried-out treatment should also have a regulatory effect on genes and proteins with a key role in the neoplastic process and lead to the opening of their expression pathways which are activated by them [17].
on the same or close to the physiological level. Due to this, in the case of this work, we decided to assess the changes in the expression of SEMA3F under the influence of cisplatin, as well as to analyze if the molecular effect, measured by the changes in expression of SEMA3F, of cisplatin is determined by its dosage or by the amount of time it was exposed to the drug. By using the commercially available endometrial cancer cell line Ishikawa [22], we were able to analyze the direct effect of the drug, isolated from the effects of the outside factors, on the expression profile of SEMA3F, not only on the transcriptome but also proteome level.

The results we gained are concurrent with the observations made by other research teams, as in cells not treated by cisplatin, the expression of SEMA3F was significantly higher than in the culture exposed to the drug [6]. Jiang et al. indicate that SEMA3F in physiological conditions functions as a suppressor of the carcinogenic induction process [23]. We observed that using the smallest dosage of cisplatin in this study already caused an increase in the expression of SEMA3F which suggests that there is a therapeutic benefit during treatment even in small doses. The conclusion from this seems evident when you consider the fact that molecular changes appear before phenotypic ones [24]. Similarly, noting the changes on the molecular level after a relatively short time of exposition of the endometrial cancer cells to the drug indicates that the effects on the phenotypic level will be evident in a later period. This also shows that it’s valuable to implement a routine diagnosis and therapy of the molecular signs, as, based on them appearing first, it is possible to predict the best treatment available [25]. Therefore, the modern approach to medicine should also be concentrated on finding new supplementary molecular markers, determining their referential range of concentrations and implementation for in vivo markers.

In our previous studies, we focused on determining the influence of directed molecular therapy (tumor necrosis factor - α, TNF- α paths and adalimumab) on the expression profile of genes of signal pathways with a key role in psoriasis pathogenesis [24]. We concluded that along with the increase in incubation time of the cells with the drug, its influence, expressed by the number of differentiated ID mRNA in the culture with adalimumab, in comparison with the control, undergoes a gradual increase. However, when speaking about the observations made in this study, we did not conclude that increasing the exposition time for endometrial cancer cells to cisplatin resulted in a change in the expression profile of SEMA3F mRNA and SEMA3F protein towards the one noted for the control culture (cells not treated by the drug). This observation is also substantial from the point of view of the aforementioned loss of responsiveness to the treatment during the use of cisplatin. Galluzzi et al. indicate that the appearance of resistance to cisplatin treatment is characterized by being complex and dependent on many factors, indicating that genetic and epigenetic factors have a key meaning. Similarly, in order to fight against the loss of the therapeutic effect of cisplatin, it would be worth having a multidimensional strategy [26]. One of the potential mechanisms which may affect the lack of any resistance to cisplatin by the cells of cell line Ishikawa being observed could be connected with the lack of induction by the adapta-
genes than in the case of using all of these drugs separately. Shen et al. noted, above all, a decrease in the expression of TGF-β1, Smad3, interleukin 2 (IL-2) and TNF-α as well as overexpression of Smad7 in comparison to the control [35].

Proof that the detection of changes in the expression profile of SEMA3F may constitute a promising diagnostic-therapeutic marker is also constituted by the observations of Nguyen et al. who confirmed, that a lowered expression level of SEMA3F in the cases of endometrial cancer [36] or also the observations of Scheerer et al. indicating, that previous inductions of SEMA3F were through inflammation [37]. Above all, in our previous study, we analyzed the differences in the expression pattern of SEMA3F in biopsies obtained from female patients with a diagnosed endometrial cancer at different stages of its histological advancement and compared to a control. We observed that, in neoplastic samples, the level of SEMA3F was substantially statistically lower than in the control [38]. Therefore, the expression pattern of SEMA3F noted by us in this study confirms that a beneficial, expected phenomenon during pharmacotherapy is an increase in the expression of SEMA3F. Based on our observations, it seems that cisplatin therapy could accompany an increase in the concentration of estrogens. It was confirmed that the endometrial cancer cell line Ishikawa shows an ability to express estrogens [39, 40]. Likewise, it indicates that estrogens are an essential way to answer for an increase in SEMA3F expression [41, 42]. Furthermore, another possible reason for determining an increase in the expression of SEMA3F during the use of cisplatin can be induced by hormonal drug changes.

Moreover, our observations confirm that not only transcripts but also proteomes are dynamic systems, which react quickly to changes. The performed analysis also confirm the apoptotic effect of cisplatin on endometrial cancer cells and the influence of the drug on SEMA3F- signaling paths.

CONCLUSION
Analysis of the changes in the expression of SEMA3F on the mRNA and protein level in endometrial cancer in vitro model indicates that using cisplatin is an effective therapeutic option in this type of neoplasm. Adding it to a culture, no model indicates that using cisplatin is an effective therapeutic marker is also constituted by the observations of Scheerer et al. who confirmed, that a lowered expression level of SEMA3F in the cases of endometrial cancer [36] or also the observations of Shen et al. indicating, that previous inductions of SEMA3F were through inflammation [37].

AVAILABILITY OF DATA AND MATERIALS
The data will not be shared due to the fact the third-party rights and commercial confidentiality.

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

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