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Critical role of SEMA5A expression in invasion and metastasis of ovarian cancer cell

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Abstract

Semaphorins are a large family of genes involved in the development and morphogenesis of the nervous system. SEMA5A has been reported as a bi-functional molecule, acting as both oncogene and tumor suppressor in different types of cancer. High expression levels of SEMA5A and its receptor, Plexin-B3, were associated with aggressiveness in pancreatic and prostate cancers. Our previous study in ovarian cancer metastasis indicates that FAK knock-down can suppress ovarian cancer cells migration and invasion. We hypothesized that SEMA5A expression promotes ovarian cancer invasion and metastasis. We investigated the expression of SEMA5A in patients with metastatic ovarian cancer (n = 43), localized tumor (n = 37) and normal ovarian tissue (n = 12) from non-malignant diseases as control with different histopathological characteristics. For Silencing of SEMA5A *in vitro*, we treated human ovarian cancer cells (OVCAR-3, A2780/CP70) with miR-27a and miR-27b. We observed significantly higher expression of SEMA5A protein (P= 0.001) in metastatic ovarian cancer tissue associated with poor overall survival outcomes compared to localized ovarian cancer and control. *In vitro* silencing of SEMA5A reduced migration and invasion of ovarian cancer cell. Our data offer opportunities for the therapeutic modulation and biomarker of metastatic ovarian cancer.

Key words: Semaphorin, SEMA5A, Plexin-B3, FAK, Ovarian cancer

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Introduction

Semaphorins are a large family of genes involved in the development and morphogenesis of the nervous system. SEMA5A has been reported as a bi-functional molecule, acting as both oncogene and tumor suppressor in different types of cancer. High expression levels of SEMA5A and its receptor, Plexin-B3, were associated with aggressiveness in pancreatic and prostate cancers. Previous study in ovarian cancer metastasis indicates that FAK knock-down can suppress ovarian cancer cells migration and invasion [1].

Previously, a comprehensive analysis of the gene expression signature performed by the authors in non-smoking women with lung adenocarcinoma revealed that the downregulation

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of *SEMA5A* was associated with a poor overall survival [2]. SEMA5A belonging to class V of the semaphorin family, is an integral membrane protein containing the Sema domain, 7 thrombospondin type-1 repeats and a short cytoplasmic domain ([3]. SEMA5A has been reported to have both a membrane-bound [4] and cleaved extracellular domain [5]. Sheddases, which are the members of the ADAM protein families, are known to be majorly involved in ectodomain shedding by cleaving the extracellular portions of transmembrane proteins [6]. However, the role of sheddase responsible for releasing the ectodomain from membrane-bound SEMA5A has yet to be identified.

In the present study, we tested the hypothesis that knockdown of SEMA5A expression can modulate tumor growth and metastasis in ovarian cancer cell. Towards this end, we performed *in vitro* and *in vivo* studies to understand the role of SEMA5A on ovarian cancer cell metastasis by generating SEMA5A knockdown cells in ovarian cancer cell lines. In contrary to our expectation, loss of SEMA5A enhanced metastatic potential of the ovarian cancer cell by mediating epithelial to mesenchymal transition.

Materials and Methods

Cells and cell culture

Human ovarian cancer cells (OVCAR-3, A2780/CP70) were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific) with 1% streptomycin/puromycin (Biological Industries) and 10% fetal bovine serum (FBS; Biological Industries). The cultured plates were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Endogenous expression of SEMA5A

To quantify the transcriptional expression of *SEMA5A* in different cellular models, total RNA was isolated using TRIzol reagent (Ambion) and purified by precipitation with isopropanol (Sigma-Aldrich). A NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific) was used to assess the purity and quantity of the RNA. A high Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) was used to synthesize the cDNA from 1 μg of total RNA of each cell line. The final cDNA products were used as the templates for expression analysis using quantitative PCR (qPCR).

qPCR

The quality and quantity of the RNA were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific). A total of 1 μg of total RNA from each cell line was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). The reaction was carried using thermal cycling conditions, including annealing at 25°C for 5 min, an extension temperature at 42°C for 1 h and inactivation temperature at 70°C for 15 min. The final cDNA products were used as the templates for subsequent qPCR with the following thermal cycling conditions. Denaturation temperature at

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95°C for 15 sec, anneal/extend temperature: 60°C for 1 min for 40 cycles. RT-qPCR was performed using SYBR-Green (Roche) on an ABI 7900 system (Life Technologies; Thermo Fisher Scientific) according to standard protocols.

Western blot analysis

Total cell lysates of A549 and H1299 cells transfected with pZeoSV2^{+}-*SEMA5A*-Flag plasmid or empty vector were prepared. Proteins (30 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories). After blocking with 5% milk, the membranes were incubated with anti-SEMA5A (1:1,000, Cat. no. PA5-26066, Thermo Fisher Scientific), anti-GAPDH antibody (1:20,000, Cat. no. GTX100118, GeneTex), or anti-BSA antibody (1:5,000, Cat. no. GTX79812, GeneTex). Following incubation overnight at 4°C, the membranes were then incubated with horseradish peroxidase-conjugated anti-IgG (1:5,000, Cat. no. GTX213110, GeneTex) at room temperature for 1 h, and the blots were developed with the chemiluminescent western blotting reagent (Millipore). Western blot images were further analyzed using Gel-Pro Analyzer v6.3 software (Meyer Instruments) to obtain the optical density values of SEMA5A and GAPDH antibodies.

Cell migration

Migration assays were carried out using a 24-well Transwell unit (Corning, Inc.). The upper chamber of the Transwell unit was loaded with $4{\times}10^4$ cells/well in 0.2 ml serum-free RPMI-1640 medium, and the lower chambers were loaded with 0.6 ml RPMI-1640 containing 10% FBS as a chemoattractant. The A549 and H1299 cells were then incubated for 24 h at 37°C. A methanol-acetic acid (3:1) mixture was then added to the lower chamber to fix the cells for 20 min at room temperature followed by staining with 0.1% crystal violet for a further 20 min at room temperature. Cells on the upper side of the membrane surface were removed by scraping with a cotton swab, and the cells that passed through the filter were de-stained using 10% acetic acid. The absorbance was measured at 570 nm with an enzyme-linked immunosorbent assay (ELISA) reader (BioTek Instruments). Cells transfected with empty vector were used as the controls.

Time-lapse migration assay

The experiment was performed as previously described [7]. Briefly, cells were cultured on dishes coated with collagen (10 μg/ml) overnight and then cultured in serum-free conditioned medium. Cell movement was detected under A-Plan objectives (X5; 0.55 NA) using an inverted microscope (Axio Observer Z1, Zeiss) in 37°C chambers. Images were collected from CCD video cameras (AxioCam MRm, Zeiss) at 20-min intervals for a total of 16 h using MetaMorph software (Molecular Devices Corp.). The accumulated distance was measured by tracking each

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cell nuclei for 30 individual cancer cells in each group using the Track Point function of NIH ImageJ v1.43 software.

Analysis of cell proliferation

A total of 4,000 ovarian cancer cell were seeded in 96-well plates in triplicate and incubated for 12 h at 37°C in a CO_2 incubator. The day after seeding, the cells were transfected with pZeoSV2^{+}-*SEMA5A*-Flag plasmid or empty vectors. Following transfection, the cells proliferative activity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (EMD Biosciences) assay at 24, 48 and 72 h, respectively. The absorbance of the A549 and H1299 cells was then measured using a microtiter plate reader (BioTek) at 570 nm.

Kaplan-Meier survival analysis

Kaplan-Meier survival analyses were conducted using Kaplan-Meier Plotter for ovarian cancer cell [8]. The log rank test was used to determine differences in the survival rate between the high and low expression groups.

Statistical analysis

The significance of the data was determined by the Student's *t*-test (two-tailed) for all in vitro studies. Comparisons between different mice groups were evaluated using Mann-Whitney U-test. The $p < 0.05$ was deemed significant. All statistical analyses were done using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

Results

Association of SEMA5A expression in ovarian cancer cell with a poor overall survival

The survival of ovarian cancer cell patients in the new dataset for >15 years was examined using Kaplan-Meier. In total, 80 patients were used for survival analysis, patients were divided into the high and low expression groups based on the median expression value of *SEMA5A*. The results of Kaplan-Meier survival analysis revealed that the ovarian cancer cell patients with higher expression levels of *SEMA5A* had a lower risk of death (hazard ratio, 0.65), i.e., a higher overall survival probability (Fig. 1B), indicating the potential utility of *SEMA5A* as a prognostic marker for patients with ovarian cancer cell.

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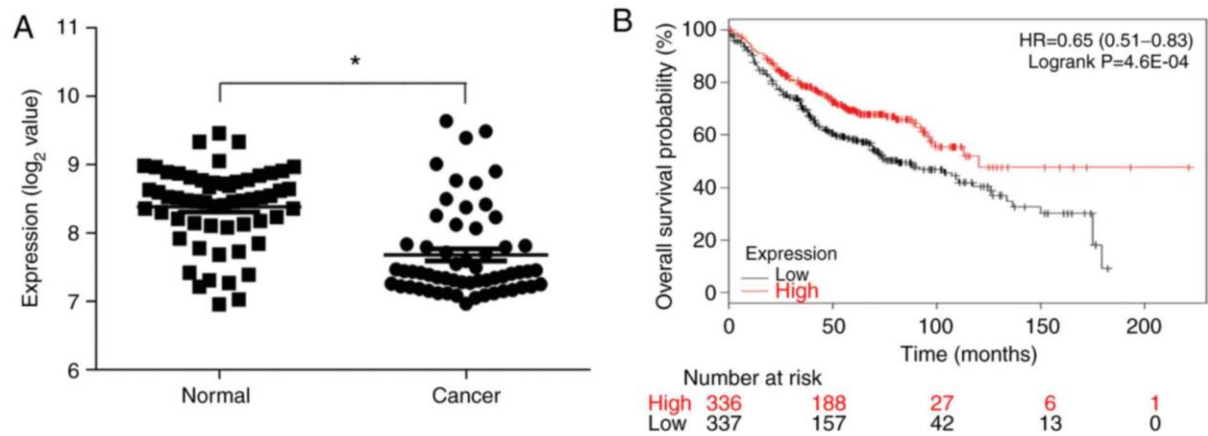

Figure 1.

Downregulation of SEMA5A ovarian cancer cell is associated with poor overall survival. (A) Expression levels of SEMA5A were examined in normal and ovarian cancer cell *P<0.01. (B) Survival analysis was performed based on the expression levels of SEMA5A using a Kaplan-Meier plotter. Patients were divided into the high and low expression groups based on the median value of all samples. P-values were calculated using a log-rank test. HR, hazard ratio; SEMA5A, semaphorin 5A.

SEMA5A knockdown induces a mesenchymal phenotype

We knocked down SEMA5A expression in T3M-4 (Fig. 1a-b) and CD18/HPAF (Fig. 1c) cells using short hairpin (sh)RNA-mediated gene silencing. Reduced expression of *SEMA5A* at the RNA (Fig. 1a), as well as the protein levels in T3M-4-shSEMA5A (Fig. 1b) and CD18/HPAF-shSEMA5A cells (Fig. 1c) in comparison with their respective non-targeting Control, were observed. To our surprise, we found a marked difference in morphology between T3M-4-shSEMA5A and -Control cells. T3M-4-Control cells were epithelial and exhibited cobblestone-like appearance with closely opposed cell-cell junctions (Fig. 1d). In contrast, T3M-4-shSEMA5A cells showed relatively elongated morphology (Fig. 1d). We observed similar changes in morphology of CD18/HPAF cells (Fig. 1e) upon knockdown of SEMA5A. CD18/HPAF-Control cells formed tight, compact overlapping cellular colonies in comparison with their respective CD18/HPAF-shSEMA5A cells, which showed flat spindle-like structures (Fig. 1e).

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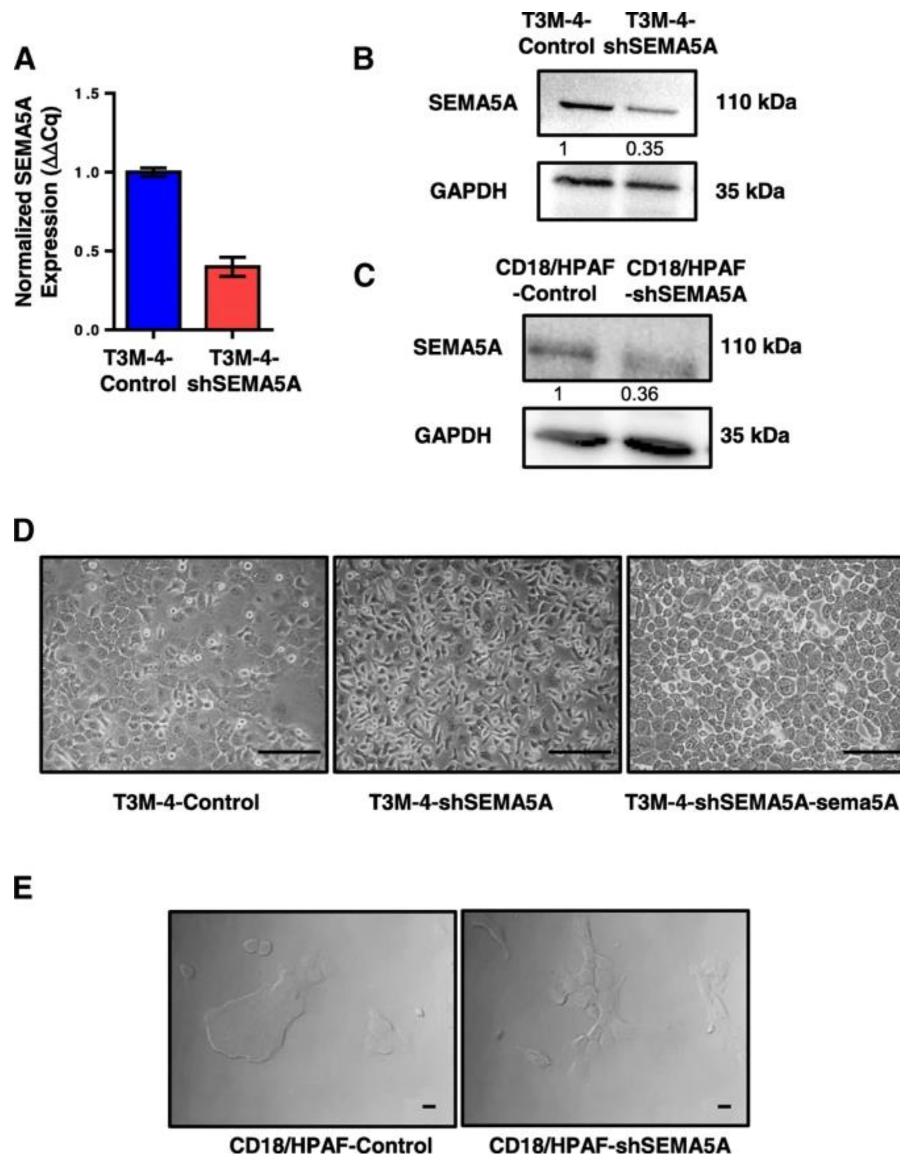
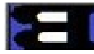


Figure 2.

Confirmation of SEMA5A knockdown in PC cells and resulting mesenchymal morphology. **a**. Bar graph showing relative fold decrease in mRNA expression of *SEMA5A*. *HPRT* is used as a control by RT-PCR analysis. The values are mean relative fold changes ± Standard Error of Mean (SEM, bars). **b**-**c**. Western blot analysis of cell lysates of T3M-4-Control and T3M-4-shSEMA5A cells (**b**) and Control and CD18/HPAF-shSEMA5A cells (**c**) showing decreased SEMA5A protein expression with GAPDH as a loading control. Quantification of the protein of interest by the intensity of the bands with respect to their loading control was performed by Image J software. Bands were normalized on the T3M-4-Control cells or CD18/HPAF-Control cells. **d**-**e** Morphological changes upon SEMA5A knockdown. Cobblestone-like T3M-4-Control cells change to spindle-shaped cells in T3M-4-shSEMA5A cells. Furthermore, on expressing Flag-tagged mouse sema5A in T3M-4-shSEMA5A cells, the cells reverted back to their cobblestone appearance. Scale bar represents 100 µm in length (**d**). The transition of overlapping colonies of CD18/HPAF-Control cells to flat spindle-shape cells in CD18/HPAF-shSEMA5A cells. Scale bar represents 10 µm in length (**e**)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SEMA5A knockdown enhances metastasis

To evaluate the tumorigenic potential of SEMA5A knockdown cells, we first went ahead and injected SEMA5A knockdown and Control T3M-4 cells (10^6) subcutaneously into the neck and flank regions of athymic nude, female mice ($n = 6$). Tumor growth was monitored twice weekly for three weeks. To our surprise, we did not observe a difference in tumor incidence (Additional file 2: Figure S3A), growth (Additional file 2: Figure S3B) or morphology (Additional file 2: Figure S3C) between SEMA5A knockdown and Control cells.

Next, in addition to evaluation of tumorigenic potential, we also wanted to study the metastatic potential of SEMA5A knockdown and Control cells. With this objective, we orthotopically injected T3M-4-shSEMA5A and -Control cells and CD18/HPAF-shSEMA5A and -Control cells into athymic nude mice. We sacrificed the mice orthotopically injected with T3M-4-shSEMA5A and -Control cells in 21 days post-injection and examined tumor growth and metastasis. However, we sacrificed the mice orthotopically injected with CD18/HPAF-shSEMA5A and -Control cells in 35 days post-injection and examined tumor growth and metastasis. We observed a statistically significant increase in mice weight ($p = 0.034$) (Fig. 3a), tumor weight ($p = 0.0159$, Fig. 3b), and increase in the number of liver macrometastases ($p = 0.0079$, Fig. 3c) in mice injected with T3M-4-shSEMA5A than T3M-4-Control cells.

In animals injected with CD18/HPAF cells, we observed no significant changes in mice weight (Additional file 2: Figure S4A) and tumor weight (Additional file 2: Figure S4B), but increase in the number of liver macrometastases ($p = 0.0083$, Additional file 2: Figure S4C) as well as liver micrometastasis ($p = 0.0083$, Additional file 2: Figure S4D) in mice injected with CD18/HPAF-shSEMA5A than CD18/HPAF-Control cells. Specifically, SEMA5A knockdown cells metastasized to the liver and spleen with a higher incidence in comparison with the Control cells in both T3M-4 (Additional file 2: Figure S4E) and CD18/HPAF cells (Additional file 2: Figure S4F). We also confirmed the maintenance of SEMA5A knockdown in the tumors formed by injection of T3M-4 cells by performing immunohistochemical staining of SEMA5A in tumor sections (Fig. 3d).

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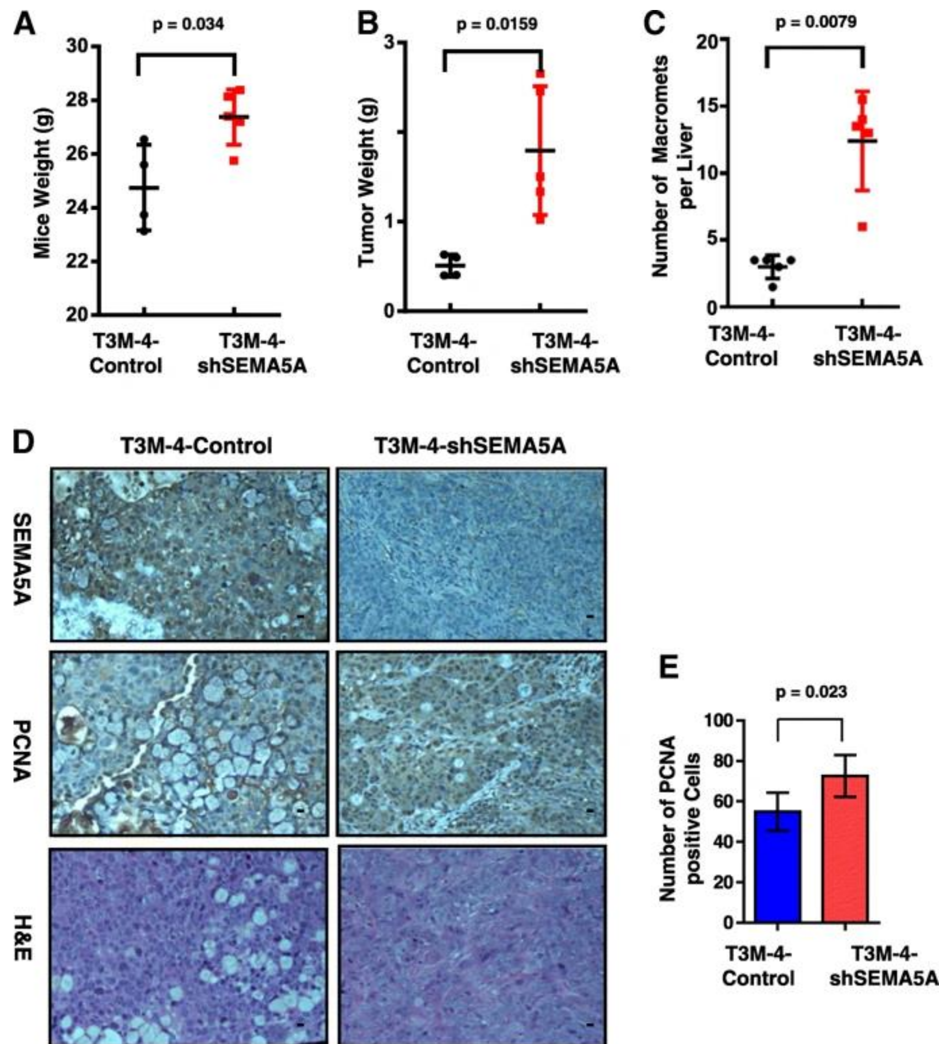

Figure

SEMA5A knockdown enhances tumor size and metastatic potential. **a**-**c** Graph showing increase in the average weight of the mice in grams ($p = 0.034$), (**a**) the average weight of primary tumor in grams ($p = 0.0159$) (**b**) and number of metastases per liver (p = 0.0079) **(c)** in mice orthotopically injected with T3M-4-shSEMA5A cells in comparison with mice injected with T3M-4-Control cells. The error bars in the graphs presented in this section represent ± Standard Deviation and significance of the data is calculated using Mann-Whitney U-Test. **d** Representative images showing SEMA5A and PCNA expression and H&E staining of orthotopic pancreatic tumors from T3M-4 shSEMA5A and Control cells. Scale bar represents 10 μm in length. Images are taken using a Nikon Eclipse E800 microscope. **e** Bar graph showing a higher number of proliferating tumor cells stained with PCNA ($p = 0.023$) in athymic mice orthotopically injected with T3M-4-shSEMA5A in comparison with those injected with Control cells. The error bars in the graphs represent ± SEM and * indicates statistical *p*-value less than 0.05 using Student t-test,

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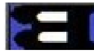 Research Article doi: 10.18081/2333-5106/014-04/247-259

Discussion

In a previous genomic study by the authors, *SEMA5A* was identified as a prognostic biomarker in non-smoking Taiwanese women with lung adenocarcinoma [8-11]. In this study, it was confirmed that the downregulation of *SEMA5A* in ovarian cancer cell was associated with a poor overall survival in different ethnic groups. In addition, lower levels of SEMA5A ovarian cancer cell were the result of both hypermethylation in the 5′untranslated region at the genetic level and cleavage to the secretory form. In addition, microarray analyses revealed that *SEMA5A*-regulated genes were involved in growth and proliferation. Finally, *in vitro* and *in vivo* analyses revealed the suppressive effects of *SEMA5A* overexpression on ovarian cancer cell in terms of proliferation, colony formation and migration.

Semaphorins represent a large family of proteins, many of which are promising targets for interfering with cancer progression due to their roles in tumor angiogenesis, tumor growth and metastasis [12-17]. In particular, previous studies have reported the roles of SEMA5A in the development of several types of cancer, such as pancreatic cancer, gastric cancer, ovarian cancer and gliomas [18-22]. SEMA5A has been reported to enhance the invasion and metastasis of gastric cancer and pancreatic cancer cells [23-25] and has been shown to be associated with a poor survival in ovarian cancer [26].

In contrast to this finding, a previous investigation by the authors revealed that the incidence of ovarian cancer cell was associated with the downregulation of *SEMA5A* expression in non-smoking female ovarian cancer cell patients, and that this down-regulation was associated with a poor overall survival [27-29]. To investigate these seemingly opposing roles of SEMA5A in different types of cancer, this study first examined the endogenous expression levels of *SEMA5A* in ovarian cancer cell. Consistent with the expression pattern in clinical tissues, the expression of *SEMA5A* was downregulated in lung adenocarcinoma cells as compared to their normal counterparts.

Furthermore, the inactivation of several tumor suppressor genes has been shown to be partly due to hypermethylation within the promoter region [30]. Epigenetically disrupted gene expression can further alter various cancer-related processes, such as cell proliferation, apoptosis and angiogenesis [31]. The abnormal DNA methylation of genes may be associated with clinical outcomes in lung cancer patients [32]. This study found that hypermethylation in the upstream genetic loci was partly responsible for the downregulation of *SEMA5A* in ovarian cancer cell, as has been previously reported for other tumor suppressor genes [33].

In this study, when the cells were treated with the methylation inhibitor, 5-aza, the upregulation of *SEMA5A* was observed only in the cancer cell lines. Pyrosequencing results further identified the methylated CpG sites modulating the expression of *SEMA5A* in these cell lines, suggesting that aberrant methylation changes result in the inactivation of *SEMA5A* in ovarian cancer cell. However, additional studies using larger numbers of tissue samples that contain clinical features are required to validate whether methylation changes of *SEMA5A* are involved in tumorigenesis.

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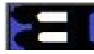 Research Article doi: 10.18081/2333-5106/014-04/247-259

Furthermore, this study demonstrated that SEMA5A can be possibly cleaved by ADAM17, which belongs to the protein family of disintegrins and metalloproteases. ADAM17 is involved in the release of a soluble ectodomain from membrane-bound pro-proteins. It has also been reported to be upregulated in non-small cell lung cancer [34], and this upregulation of ADAM17 can be caused due to ionizing radiation. Moreover, the silencing of *ADAM17* has been shown to suppress the migration and invasion of A549 cells *in vitro*, and tumor growth *in vivo* [35]. Since previous studies have demonstrated that the mature form of SEMA5B is proteolytically processed by ADAM17 [36], and that the cleaved extracellular domain of SEMA5A decreases following the silencing *ADAM17* [37], this study assessed the proteolytic effect of recombinant ADAM17 on SEMA5A. The results suggested that the membrane-bound SEMA5A was exported to the medium following cleavage by ADAM17. However, further clarifications are required to conclude whether the endogenous ADAM17 in ovarian cancer cell has enough proteolytic activity to shed membrane-bound SEMA5A.

The extracellular domain of SEMA5A is involved in angiogenesis [38]. There is evidence to indicate an increase in proliferation and the upregulation of anti-apoptotic genes (e.g., *BCL-2* and *BIRC5)* following treatment of endothelial cells with the extracellular domain of recombinant SEMA5A [39]. In addition, pancreatic cells transfected with the extracellular domain of SEMA5A exhibit a greater metastatic potential and an enhanced endothelial cell proliferative ability [40]. These results suggest that the extracellular domain of SEMA5A plays a potential role in carcinogenesis.

However, this study found that the total amount of membrane-bound SEMA5A in ovarian cancer cell was downregulated compared to normal ovarian cells. The transient overexpression of *SEMA5A* in ovarian cancer cell had tumor-suppressive effects, such as decreasing cell proliferation, colony formation and migration, although not increasing apoptosis. Furthermore, lower expression levels of *SEMA5A* were found to be associated with a worse prognosis, which suggested the tumor suppressive role of SEMA5A in ovarian cancer cell.

Consistent with the findings of this study, lower endogenous SEMA5A levels have been found to be associated with increased invasiveness in glioma. Furthermore, SEMA5A has been shown to impede the motility of human gliomas upon its interaction with Plexin-B3 via the indirect inactivation of Rac1 through RhoGDIα, and the inactivation of protein kinase C (PKC) to phosphorylate fascin-1 [41]. However, the transcriptional levels of *RAC1* and *FSCN1* did not alter significantly in this study, and whether the levels and activity of these proteins are altered remains to be determined in lung adenocarcinoma cells.

On the contrary, a high expression of SEMA5A protein has been shown to be associated with poor overall survival outcomes in metastatic ovarian cancer [19] and to be associated with progression and metastasis in gastric cancer and pancreatic cancer [11]. In explaining the discrepancy of the functions of SEMA5A, it was thus speculated that the cleaved extracellular domain and full-length of SEMA5A may carry out opposite functions in different cancer types. The alternative explanation is that receptor-ligand interactions generate simultaneous

bidirectional signals (i.e., forward signaling and reverse signaling) with opposite functions [8]. That is, the full-length of SEMA5A on the membrane may function as both a receptor and ligand to generate simultaneous forward and reverse signals, whereas the cleaved extracellular domain may only initiate reverse signaling by serving as ligand for receptors on other cells. Therefore, it was hypothesized that these phenomena may mainly be due to different experimental settings and tumor types. Yet, further studies are warranted to explore the function of SEMA5A in different cancer types.

Finally, the functions of *SEMA5A* in ovarian cancer cell were investigated by identifying the downregulated related genes using microarrays. Both pathway analysis and network analysis revealed that one function of *SEMA5A*-regulated genes was cell growth and proliferation. Among these genes involved in growth and proliferation, a number of genes have been reported with similar functions in other types of cancer. For example, *ARRDC3* and *CASP1* were found to be upregulated in this study. *ARRDC3* has been reported to suppress breast carcinoma invasion [4].

The downregulation of the expression of *CASP1* has been shown to result in the proliferation and invasion of breast cancer cells [9]. In this study, the functions of SEMA5A were further validated in *in vitro* and *in vivo* experiments, demonstrating that SEMA5A truly plays a tumor-suppressive role in the proliferation and migration of lung adenocarcinoma cells. On the whole, the findings of this study may thus contribute to the development of novel therapeutic regimens for ovarian cancer cell in the future.

Conclusions

Our data offer opportunities for the therapeutic modulation and biomarker of metastatic ovarian cancer.

Competing interests

The authors declare that they have no competing interests.

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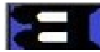
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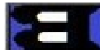
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