Pancreatic adenocarcinoma up-regulated factor as a target for the treatment of serous ovarian adenocarcinoma

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Abstract

Ovarian cancer is the deadliest gynecological cancer and when diagnosed at advanced stages the five-year survival rate is typically less than 30%. Pancreatic adenocarcinoma up-regulated factor (PAUF) plays a crucial role in tumor progression, angiogenesis, and immune evasion in the pancreatic tumor microenvironment. The current study aimed to characterize the potential tumorigenic role of PAUF in serous ovarian cancer as high expression of the factor is correlated with poor prognosis in epithelial ovarian cancer. Extracellular treatment with recombinant PAUF activated intracellular signal pathways that subsequently led to enhanced proliferation, migration, and invasion in various serous ovarian adenocarcinoma cell lines. A PAUF-knockout OVCAR-5 cell line demonstrated an apparent reduction in tumor incidence and delayed tumor growth in mouse xenograft experiments. Furthermore, the administration of an anti-PAUF antibody exhibited a notable therapeutic efficacy in mice bearing the OVCAR-5 cell line-derived xenograft tumors. Taken together, our results suggest that PAUF plays a role in the progression of serous ovarian adenocarcinoma and may serve as a novel therapeutic target of the disease.

Introduction

Ovarian cancer is the deadliest gynecological cancer, and as so few symptoms appear in the early stages, more than 70% of patients are diagnosed at advanced stages [1, 2]. The five-year survival rate of the patients with advanced stage ovarian cancer is less than 30%. Epithelial ovarian cancer is the most common type of ovarian cancer and serous adenocarcinoma, which accounts for 75 ~ 80% of the epithelial type is an aggressive disease [3]. Current treatments for serous ovarian cancer include volume-reduction surgery and platinum-based chemotherapy; the primary drugs used are cisplatin or a carboplatin and paclitaxel regimen [4]. However, the effects of these chemical drug therapies have been reported to be insignificant with poor patient outcomes due to drug resistance of ovarian cancer cells and rapid tumor recurrence [5, 6]. Thus, there remains a need for better diagnostic tools, treatment interventions, and novel biomarkers for serous ovarian cancer.

We have previously reported that pancreatic adenocarcinoma up-regulated factor (PAUF) is a secreted metastasis factor in the pancreatic tumor microenvironment [7]. PAUF is overexpressed in pancreatic ductal adenocarcinoma (PDAC) and exerts its cellular functions in both autocrine and paracrine manners. PAUF acts on: i) pancreatic cancer cells to promote tumorigenic progression and metastasis [7–11], ii) endothelial cells to induce angiogenesis in a tumor mass [12], and iii) immune cells to evade immune surveillance in the tumor microenvironment [13, 14]. Recent reports indicate that PAUF is overexpressed in ovarian cancer [15, 16], which is consistent with our previous observation that the PAUF transcript is elevated in the disease [7]. PAUF expression is associated with tumor grade and chemoresistance of epithelial ovarian cancer [15, 16]. Furthermore, high expression of PAUF correlates with poor prognosis for both overall and progression-free survivals in epithelial ovarian cancer. However, the role of PAUF as a biomarker and/or therapeutic target in ovarian cancer remains unclear.
The current study aimed to identify the function of PAUF in serous ovarian cancer using cell-based assays and mouse xenograft experiments. Findings indicate that PAUF is a useful biomarker and a novel therapeutic target for the treatment of serous ovarian cancer.

**Materials And Methods**

**Cell culture and reagents**

Human serous ovarian adenocarcinoma cell lines (HeyA8, A2780, SK-OV-3, OVCAR-5, OVCAR-8) were obtained from the National Cancer Center of Korea. The Lenti-XTM 293T cell line was obtained from Clontech. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and cultured in the medium recommended by the suppliers: Roswell Park Memorial Institute (RPMI) was used for HeyA8, A2780, SK-OV-3, OVCAR-5 and OVCAR-8 and Dulbecco's modified Eagle's medium (DMEM) was used for Lenti-XTM 293T. Culture media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/ml streptomycin, and 100 IU/ml penicillin. RPMI, DMEM, and FBS were purchased from GE Healthcare Life Science. Cells were periodically observed by monitoring cell morphology and growth rates, and mycoplasma contamination was monitored using a mycoplasma detection kit (Lonza). Authentication of the cell lines was done using short tandem repeat (STR) profiling by the National Cancer Center of Korea with proper STR references. Recombinant PAUF protein was prepared as previously described [8]. Humanized anti-PAUF monoclonal antibody was prepared as described (KR Patent 10-1856904, 2018). Human immunoglobulin G (Thermo Scientific) was used as a control for IgG. Antibodies against ERK, p-ERK, Src, p-Src, AKT, p-AKT and Cas9 were obtained from Cell Signaling Technology. Anti-β-actin antibody was obtained from Santa Cruz Biotechnology.

**Generation of PAUF-knockout ovarian cancer cell line**

A PAUF-knockout OVCAR-5 cell line was generated using CRISPR/Cas9 genome editing. For sgRNA, we used the CRISPR/Cas9 design tool (http://crispr.mit.edu) to select key regions of the genome for protein function and to search for sgRNA [17]. Target specificity of the sgRNA sequence to PAUF was verified by using the BLAST search in NCBI. The oligonucleotides of the sgRNA sequence (5’-CACCGGACTACGACCATGAAATCAC-3’ and 5’-AAACGTGATTTCATGGTCGTAGTCC-3’), were annealed and inserted into the BsmBI (Enzynomics) site of LentiCRISPR vector (Addgene). The mock control sequence (5’-GTTCCGCGTTACATACTTA-3’) had no other genomic matches [18]. Confirmation of transduction was performed by quantitative reverse transcription real-time polymerase chain reaction (RT-qPCR) and Western blot analysis of Cas9. The primer sequences for Cas9 RT-qPCR were 5’-GGACTCCGGATGAACTA-3’ and 5’-TCGCTTCAGCTTAGGA-3’.

**Proliferation assays**

Proliferation assays were performed using AlamarBlue (Invitrogen) and WST-1 (Roche). The AlamarBlue assay was carried out according to manufacturer's instructions. Briefly, cells were seeded 1 x 10³ cells per well in 96-well plates and fluorescence was quantified using a microplate reader. The WST-1 assay was
carried out according to manufacturer’s instructions. Briefly, cells were seeded 3 x 10^3 cells per well in 96-well plates and absorbance was quantified using a microplate reader. Three independent experiments were performed with duplicate samples in each. Data are presented as mean ± SEM of three independent experiments, and representative images are shown. *P < 0.05.

**Migration & invasion assays**

Migration and invasion assays were performed in Transwell plates purchased from Corning. For the invasion assay, detached cells were seeded in the upper chamber coated with Matrigel (BD Biosciences) in serum-free culture medium. The lower wells of the chamber were filled with standard medium and cells were seeded into upper chambers containing recombinant proteins or antibodies. After incubation for 20 hours at 37˚C, the cells on the upper chamber of the membrane were completely removed with a moist cotton swab. For both assays, the cells were fixed with methanol and stained, then counted and photographed by microscopy at x100 magnification. All assays were performed in duplicate and the tendency was confirmed by three independent experiments. Data are presented as mean ± SEM of three independent experiments, and representative images are shown. *P < 0.05.

**Adhesion assays**

Adhesion assays were performed using a 24-well plate coated with collagen type I (Sigma-Aldrich). Cells were seeded in each well and unbound cells were removed by washing twice with PBS. Cell adhesion was quantified by counting the number of stained cells. All assays were performed in triplicate and the tendencies confirmed with three independent experiments. *P < 0.05.

**Sandwich ELISA**

For preparation of secreted PAUF, cells were cultured for three days after seeding 7 x 10^5 cells followed by concentration of culture supernatant using a Vivaspin column (Sartorius) per manufacturer’s recommendation. Detection of PAUF by ELISA was as described previously [16].

**Ethical approval**

All animal studies were performed according to the protocol (2018-12-187, 2019-12-176) approved by Institutional Animal Care and Use Committee of Asan Institute for Life Sciences, Korea.

**In vivo animal experiments**

The OVCAR5 cell-derived xenograft tumor models made of female athymic nude mice (BALB/c nu/nu; 6 weeks old; Japan SLC, Hamamatsu, Japan) were used for the examination of the in vivo test. For the tumorigenesis test, OVCAR-5 or its derivative cells were cultured and resuspended in saline. Approximately 1 x 10^6 cells were subcutaneously implanted into the right hind leg of BALB/c nu/nu mice. For the in vivo efficacy test, the mice were implanted and allowed to rest for a period of ~8 days to allow the approximate average tumor volume to reach 100 mm^3 before treatment. Groups were randomized,
with 5~15 mice in each experimental group. A control IgG and anti-PAUF antibody at a dose of 10 mg/kg was given intravenously. Mice were treated with the dose twice a week (3-4 day intervals) for four weeks. Docetaxel (Sanofi-Aventis) was administered intravenously once for four weeks at a dose of 10 mg/kg. Tumor volume was calculated by measuring 2 perpendicular diameters with a caliper and using the formula of V= (a^2 x b)/2 where, a and b are the short and long diameters, respectively. Mice that reached the euthanasia criteria were euthanized by carbon dioxide (CO\textsubscript{2}) inhalation. Body weight was measured twice a week (3-4 day intervals) on the day of tumor measurement. Data for tumor and body weight were plotted to indicate the tumor growth curve and body weight change. Survival was analyzed by the Kaplan-Meier method, and comparisons between groups were analyzed by the Wilcoxon and log-rank tests.

**Statistical analysis**

Data generated from at least three experiments performed in triplicate were analyzed using the two-tailed Student’s t-test. Results were considered significant when \( P < 0.05 \).

**Results**

**PAUF enhances the migration and invasion of human serous ovarian cancer cells *in vitro***

To determine the endogenous levels of PAUF expression in various human serous ovarian adenocarcinoma cell lines, cells were cultured and a sandwich ELISA of culture supernatants was conducted. Levels of PAUF were highest in OVCAR-5 cells (~19 ng per 1 x 10\textsuperscript{6} cells) while HeyA8, A2780, SK-OV-3, and OVCAR-8 cells showed modest expression (Fig. 1a). PAUF expression in OVCAR-5 was higher when compared with CFPAC-1, the pancreatic cancer cell line previously shown to express PAUF at high levels [19] (Fig. 1b). Next, the potential impact of PAUF on the motility and invasiveness of serous ovarian cancer cell lines was characterized. The three cell lines with a modest PAUF expression-HeyA8, SK-OV-3, and OVCAR-8-were treated with recombinant PAUF (rPAUF). As shown in Fig. 1c-f, migration, invasion, and adhesion activities increased significantly in the treated cells when compared to the control cells (i.e., no PAUF). These results are consistent with our previous findings in pancreatic cancer cell lines [8, 10]. However, the treatment did not show significant changes in the motility and invasiveness of the OVCAR-5 cell line, which expresses PAUF at a high level. Treatment of OVCAR-8 cells with rPAUF activated intracellular signaling pathways involving ERK, Src, and AKT (Fig. 1g and Fig. S1), implying that PAUF may exert similar functions in both ovarian and pancreatic cancer cells [8-10]. These results suggest that PAUF may function as a factor that affects the metastasis in serous ovarian adenocarcinoma.

**Reduced PAUF activity diminishes migration, invasion, and proliferation of serous ovarian cancer cells *in vitro***

We explored the effects of an antibody that neutralizes PAUF function. The anti-PAUF antibody significantly inhibited the migration and invasion of OVCAR-5 cells (Fig. 2). The inhibitory activity of the anti-PAUF antibody was slightly less in cell lines with lower PAUF expression (i.e., HeyA8, SK-OV-3, OVCAR-8). To determine whether cancer-related cellular activities could be attenuated by reducing the
level of PAUF, we established a stable PAUF-knockout OVCAR-5 cells line (OVCAR-5_PAUF K/O as described in the Materials and Methods). Successful transduction was confirmed by RT-qPCR and immunoblot analysis (Fig. 3a, b and Fig. S2). OVCAR-5_Mock cells were established as a negative control. As determined by sandwich ELISA, the level of PAUF expression in the OVCAR-5_PAUF K/O cells was reduced by more than 90% compared to the OVCAR-5_Mock cells (Fig. 3c). PAUF K/O cells exhibited significantly lower migratory and invasion capabilities when compared to the control cells (Fig. 3d, e). PAUF K/O cells also showed a decrease in proliferative capacity (Fig. 3f). Treatment of PAUF K/O cells with rPAUF recovered the motility, invasion, and proliferation abilities (Fig. 3g). Therefore, both functional inhibition and expresional knockout of PAUF resulted in lowered cancer cell activities, suggesting that PAUF may be used for a targeted anti-cancer therapy to treat serous ovarian adenocarcinoma.

**PAUF-knockout delayed in vivo xenograft tumor growth**

*In vivo* relevance of the *in vitro* results was determined by evaluating the tumorigenesis and tumor growth rate in ovarian xenograft tumor models. Mice were subcutaneously implanted with OVCAR-5_PAUF K/O or OVCAR-5_Mock cells and tumor growth curves were plotted using tumor volumes measured twice a week for 46 days (Fig. 4). Individual and average tumor growth curves are shown in Fig. 4a, b, respectively. Overall, tumor growth in the OVCAR-5_PAUF K/O group was slower when compared with the OVCAR-5_Mock group. It took a mean of 32 days for tumors in the OVCAR-5_Mock group to reach 500 mm$^3$ compared with 36 days in the OVCAR-5_PAUF K/O group, indicating delayed tumor growth by PAUF-knockout. At the endpoint of the experiment, the tumor growth inhibition (TGI) percentages were calculated. On day 46, the average tumor volumes of OVCAR-5_PAUF K/O and OVCAR-5_Mock groups were 1,200 mm$^3$ and 1,800 mm$^3$, respectively, correlating to a TGI of 32.92% from PAUF-knockout (Fig. 4c). The largest and smallest volumes of OVCAR-5_PAUF K/O group were 1,300 mm$^3$ and 800 mm$^3$, while those volumes of OVCAR-5_Mock group were 2,000 mm$^3$ and 1,500 mm$^3$. These results strongly suggested that the PAUF expression was involved in ovarian tumor progression *in vivo* and could be a therapeutic target for human ovarian cancer treatment.

**An anti-PAUF antibody demonstrated therapeutic efficacy in an ovarian cancer xenograft mouse model**

We tested whether an anti-PAUF antibody is an effective treatment for human serous ovarian cancer. Mice bearing OVCAR-5-derived subcutaneous xenograft tumors were randomly divided into two groups and treated with 10 mg/kg anti-PAUF antibody or control IgG intravenously twice a week (Fig. 5a-d). The tumor growth rate and distribution of tumor volumes between IgG-treated and anti-PAUF antibody-treated groups were analyzed; average tumor growth curves are shown in Fig. 5a. The results indicate that the anti-PAUF antibody tested here is capable of reducing the growth of OVCAR-5 xenograft tumors in a mouse model. As shown in Fig. 5b, on day 23, the average tumor volumes of the anti-PAUF antibody-treated and control IgG-treated groups were 1,400 mm$^3$ and 1,800 mm$^3$, respectively, reflecting a 27% TGI effect of treatment with an anti-PAUF antibody. A Kaplan-Meier analysis indicated that the survival rate of the anti-PAUF antibody-treated group increased 28.6% compared to that of the control IgG-treated group (Fig. 5c). There was no difference in body weight between the two groups (Fig. 5d). To test the
combination effect of the antibody with docetaxel, the tumor growth rate and distribution of tumor volumes between IgG, anti-PAUF antibody, docetaxel, and combination-groups were analyzed; average tumor growth curves are shown in Fig. 5e. As shown in Fig. 5f, on day 31 the average tumor volumes of the control IgG, anti-PAUF antibody, docetaxel, and combination treated groups were 2,600 mm$^3$, 2,200 mm$^3$, 1,700 mm$^3$, and 1,600 mm$^3$, respectively. In the current experiment, a Kaplan-Meier analysis indicated that while the anti-PAUF antibody showed a 17% improvement of the median survival rate when compared with control IgG, docetaxel alone showed 24% compared with control IgG. Furthermore, the combination of docetaxel and anti-PAUF antibody exhibited a significantly improved median survival rate of 29% (Fig. 5g). There was no difference in body weight among the test groups (Fig. 5h). Our results clearly demonstrate that the anti-PAUF antibody reduced tumor growth and improved survival in OVCAR-5-derived xenograft tumor models, suggesting that PAUF could be targeted by therapeutic antibodies for the treatment of human ovarian serous carcinoma.

Discussion

The current study provides evidence that targeting PAUF may serve as an effective modality for the treatment of serous ovarian adenocarcinoma, which is the first report of these finding to date. Here, lines of evidence are presented for the identification of PAUF as a potential therapeutic target for serous ovarian cancer. First, variations in the levels of PAUF, achieved through either knockout or exogenous treatment approaches, led to changes in tumor related-phenotypes (i.e., migration, invasion, adhesion, proliferation) of serous ovarian cancer cells. Second, the intracellular signaling pathways activated by PAUF in serous ovarian cancer cells were similar to those observed in pancreatic cancer cells [8, 10]. Third, PAUF-knockout led to delayed tumor growth in a xenograft mouse model with the serous ovarian cancer cell line OVCAR-5. Finally, treatment of mice bearing OVCAR-5-derived xenograft tumors with a PAUF-neutralizing antibody resulted in retarded tumor growth and improved survival.

Ultrasonography and cancer antigen 125 (CA125) are standard methods for the detection of ovarian cancer [20]. However, early diagnosis of ovarian cancer is limited as diagnosis can be inaccurate with ultrasonography, and CA125, which is also detected in benign gynecological diseases and other tumors, rises only in 50% to 60% in early-stage ovarian cancer patients [21]. As a result, roughly 70% of ovarian cancer patients are diagnosed with an advanced stage of the disease [1, 2]. Therefore, research to identify new tumor biomarkers that can supplement or replace CA125 are needed. Two recent publications have highlighted the clinical importance of PAUF in ovarian cancer, suggesting PAUF as a prognostic factor in epithelial ovarian cancer [15, 16]. PAUF is highly expressed in epithelial ovarian adenocarcinoma and associated with tumor grade and chemoresistance [15, 16]. PAUF expression correlates with poor prognosis for both progression-free survival and overall survival. Here, we show that PAUF is a malignant factor in the progression of serous ovarian cancer in vitro and in vivo. Taken together, we propose PAUF is a potential novel biomarker for serous ovarian cancer.

Current treatment for ovarian cancer includes surgery and platinum-based chemotherapy [4], however, the majority of patients with advanced stages develop recurrent disease and resistance to chemotherapy [5,
Many targeted treatment approaches, including biological drugs, are under development with limited clinical benefits reported to date [22]. Targeting PAUF has been shown to be effective in inhibiting pancreatic tumor growth in xenograft mouse models. A PAUF-specific aptamer or suppression of PAUF expression inhibits the progression of the tumor growth [23, 24] and enhances chemotherapy sensitivity [25, 26]. Furthermore, a PAUF-neutralizing antibody reduced tumor growth and distant metastasis [19]. Here we show that PAUF-knockout or anti-PAUF antibody limits ovarian tumor growth in xenograft mouse models, implying that targeting PAUF could be an effective therapeutic modality to treat serous ovarian adenocarcinoma as well as pancreatic ductal adenocarcinoma. Although the pathogenic differences between pancreatic and ovarian tumors are not yet clear [27], we suggest that PAUF overexpression is a common risk factor for these diseases.

Further studies should include developing a non-invasive method to detect PAUF for diagnosis and prognosis of ovarian cancer, validating the efficacy of the anti-PAUF antibody in models reflecting the heterogeneity of patients, and evaluating of the therapeutic antibody in combination with chemical drugs.

**Declarations**

**Author Contributions**

Yeon Jeong Kim and Sang Seok Koh designed the study. Yeon Jeong Kim, Jin Park, Hyeon Hee Jeong and Ji Eun Baek performed the experiments. Seung-Mo Hong, Seong-Yun Jeong and Sang Seok Koh analyzed the data. The first draft of the manuscript was written by Yeon Jeong Kim, Jin Park and Sang Seok Koh and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data Availability**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

**Compliance with Ethical Standards**

**Conflict of Interest**

The authors declare that they have no conflict of interest.

**Ethical Approval**
All animal studies were performed according to the protocol (2018-12-187, 2019-12-176) approved by Institutional Animal Care and Use Committee of Asan Institute for Life Sciences, Korea.

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