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SERPINB7 Expression Predicts Poor Pancreatic Cancer Survival Upon Gemcitabine Treatment

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Abstract

Stratification of patients with pancreatic ductal adenocarcinoma (PDAC) remains a key challenge in the field of clinical oncology. No predictive biomarkers have yet been found for any available treatment options. Previously, we identified *SERPINB7* as a putative biomarker for PDAC and thus, herein, we aimed to validate our previous findings and assessed the predictive value of *SERPINB7.* Patients who underwent surgery and received gemcitabine (gem) or gemcitabine plus nab-paclitaxel (gem/nab) as adjuvant therapy, between 2011 and 2017, were included in this study (n = 57). Expression level of *SERPINB7* was assessed in tumor tissue by immunohistochemistry (IHC) and RNA in situ hybridization (RNA ISH). Its association with disease-free survival (DFS) and overall survival (OS) was investigated. While IHC did not show any correlation between survival and the protein level of *SERPINB7*, RNA ISH revealed that expression of *SERPINB7* was associated with a poor DFS ($P = .01$) and OS ($P = .002$) in the gem group but not in the gem/nab. Adjusted Cox-regression analysis confirmed the independent predictive value of *SERPINB7* on OS ($P = .006$, HR: 3.47; 95% CI: 1.49–8.09) in the gem group. In conclusion, *SERPINB7* was identified as the first predictive RNA biomarker for PDAC. This study suggests that patients who expressed *SERPINB7* might receive another treatment than gem alone.

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Background

In the last decade, it was possible to translate groundbreaking scientific discoveries into oncologic therapies in order to improve patients´ lives. Representative examples of these breakthroughs are ipilimumab (the first anti-cancer immunotherapy) and talimogene laherparepvec (the first oncolytic virus therapy); both approved to treat metastatic melanoma [1,2]. Even if these technological and medical advances are irrefutable, not all oncologic patients have benefited from these major improvements. Pancreatic ductal

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adenocarcinoma (PDAC) represents one of these exceptions: it is projected to become the second deadliest type of cancer in the United States by 2030 [3] and among all other cancer types, only its mortality rate is predicted to increase in the European Union for both sexes [4].

Latest research studies have shown that PDAC exhibits some special features that differentiate it from other cancer types [5]. Briefly, PDAC seems not to follow the classical step-by-step cancer development model but to follow a development path similar to a 'big bang model', in which a single phenomenon called chromothripsis originates several chromosomal rearrangements [6]. Furthermore, thanks to the advancement of new technologies, such as Next Generation Sequencing (NGS), the genetic and transcriptomic landscapes of pancreatic cancer have started to be elucidated, enabling a deeper insight into the tumor biology of this complex disease. For instance, it was shown that patients with PDAC indeed exhibit recurrent gene mutations but these classical mutations (e.g., mutations in *TP53* and *KRAS*) appear together with other rare mutations, in combinations that seem to be unique for each patient [7–10]. This apparent futility of profiling somatic mutations to predict outcome was later supported by Dal Molin et al., who demonstrated that mutations at the DNA-level were not useful predictors of overall survival in a long-survival cohort [11]. Notwithstanding these discouraging findings, results of gene expression analyses have been very promising so far. More recent evidence has revealed that transcriptome analysis enables the identification of subgroups of PDAC that might have clinical implications [12–15]. Although there is a high discrepancy between the differentially expressed genes of the three classifications, it was demonstrated that there is a high concordance in predicting patient's outcome [12–15]. For example, Bailey's squamous subtype includes genes, which are involved in e.g. inflammation and metabolic reprogramming and according to unsupervised analysis of RNA-seq data, this subtype corresponds to Moffitt's basal subtype [13–15]. In both cases, a poor overall survival (OS) was associated with these subtypes [13–15]. Overall, these data strongly suggest that there is an urgent need for changing the research strategies in order to finally identify predictive biomarkers for pancreatic cancer that might improve survival and the quality of life of patients.

To date, only our and few other research groups have focused on the identification of RNA-biomarkers for pancreatic cancer [16–18]. Recently, we have performed a retrospective analysis of a small cohort of patients with metastatic PDAC who were treated with capecitabine plus nab-paclitaxel and participated in a phase II clinical trial [16]. In this study, patients were divided into short- and long-term survivors according to the median OS and we analyzed the differential gene expression between these two groups by RNA-seq [16]. These results were then validated using publically available data from the The Cancer Genome Atlas (TCGA) database, revealing that SERPINB7 (serine protease inhibitor, clade B, member 7) expression was an independent predictor of OS [16,19]. Based on our aforementioned study (see *Supplementary Methods*), we hypothesized that SERPINB7 overexpression in the primary tumor might correlate with a poor prognosis among patients who underwent surgery for tumor resection and received gemcitabine as adjuvant therapy, a treatment considered to be the standard of care for this disease. Thus, to address this issue, we examined the RNA- and protein level of SERPINB7 by RNA in situ hybridization (RNA ISH) and immunohistochemistry (IHC), respectively, and evaluated the association between SERPINB7 expression level and disease-free survival (DFS) and overall survival (OS).

Methods

Study Cohort

Patients with PDAC presented in our multi-disciplinary tumor board meeting between 2011 and 2017 were included in this retrospective study. All patients underwent surgery and received gemcitabine (n = 46) or gemcitabine plus nab-paclitaxel (n = 11) as adjuvant chemotherapy at our institution. This study was approved by the Ethics Committee of the Medical University of Vienna (1794/2017). (More information in *Supplementary Methods*).

RNA ISH

RNA ISH was performed according to the manufacturer's instructions (RNAscope 2.5 Brown Assay, Advanced Cell Diagnostics (ACD), Hayward, CA) [20]. Briefly, FFPE tissue was deparaffinized in xylene and ethanol. Samples were incubated in citrate buffer, rinsed in water and treated with protease at 40 °C for 30 minutes in a HybEZ Oven (Advanced Cell Diagnostics, Hayward, CA). After that, samples were incubated with a custom designed sample probe and then with the probes AMP1 to AMP6. Chromogenic detection was performed using 3,3′-diaminobenzidine (DAB). Sections were counterstained with diluted Gill III (EMD Millipore, Billerica, MA, HX55589174) followed by an incubation in Scott's tap water (Morphisto, Germany, 11,192.01000). Slides were dehydrated using ethanol and xylene and then mounted with Neo-Mount (MerckKGaA, Germany, HX69711216). Positive and negative control probes (Polr2A (DNA-directed RNA polymerase II subunit RPB1) and dapB (bacterial dihydrodipicolinate reductase), respectively) were acquired from ACD. The target probe was designed by our group based on our previous results and level-1 data from TCGA (access Request #59059–3) [16,21]. If dots were detected by the pathologist in tumor cells or reactive pancreatic ducts, samples were considered as positive. Stained samples were scanned using the Aperio ScanScope (Leica Biosystems) at 40× objective magnification.

Immunohistochemistry

Sections were deparaffinized and rehydrated by washing in xylene, ethanol and water. Endogenous peroxidase activity was blocked by

Table 1. Baseline characteristics of patients included in the study before surgery. All patients underwent surgery and received gem or gem/nab at our institution.

		Gemcitabine (n = 46)	Gemcitabine + nab-paclitaxel (n = 11)*
Age at diagnosis (median)		65.72 y	65.48 y
	Female	18 (39.13%)	3 (27.27%)
Gender	Male	28 (60.87%)	8 (72.73%)
	1	1 (2.17%)	0
	2	6 (13.04%)	0
	3	38 (82.61%)	11 (100%)
T	4	1 (2.17%)	0
	0	9 (19.57%)	2 (18.18%)
N	1	37 (80.43%)	9 (81.82%)
	0	41 (89.13%)	10 (90.91%)
	1	2 (4.35%)	1 (9.09%)
M	x	3 (6.52%)	0
	1	1 (2.17%)	0
	2	31 (67.39%)	6 (54.55%)
Grade	3	14 (30.43%)	5 (45.45%)
		2 (4.35%)	1 (9.09%)
	Lung	1	0
	Liver	1	0
Metastasis at diagnosis	Other	0	1
Resection of tumor		46 (100%)	11 (100%)
R	0	32 (69.57%)	8 (72.73%)

using the Dual Endogenous Enzyme Block (Dako EnVision + Dual Link System-HRP, Dako, #K4065). Antigen retrieval was performed in 0.01 M citrate buffer (pH: 6.0, 10×) (Sigma). After 20 min cooling, sections were stained for SERPINB7 (Sigma-Aldrich, Prestige Antibodies **HPA024200) and** detected using the EnVison + Dual Link kit (Dako) according to the manufacturer's instructions. Counterstaining was performed with Mayer's hemalum solution (diluted 1:5) (Merck, Germany) and mounted with Aquatex (Merck, Darmstadt, Germany). Head and neck squamous cell carcinoma samples were used as positive controls as reported in the Human Protein Atlas [22,23]. For the negative controls, the primary antibody was omitted. Slides were observed under a microscope and analyzed by a pathologist. Samples were considered as positive if immunostaining was present in the cytoplasm of tumor cells or reactive ducts. Stained samples were scanned using the Aperio ScanScope (Leica Biosystems) at 40x objective magnification.

Statistical Analysis

Disease-free survival (DFS) was defined as time from start of treatment until disease progression and overall survival (OS) was defined as time from the start of treatment until death. Patients who were still alive were censored at the date of last follow-up. R v3.4.2 was used for all statistical analysis. Cox hazard and log-rank tests were performed using the Survival v2.41–3 library, in case of invalid Cox proportional assumptions a two-stage test via the TSHRC v0.1–5 package was computed. Association of SERPINB7 expression and clinicopathological characteristics was analyzed using a Chi-square test. Kaplan–Meier curves for patient survival were plotted using GraphPad Prism 6.0 Software.

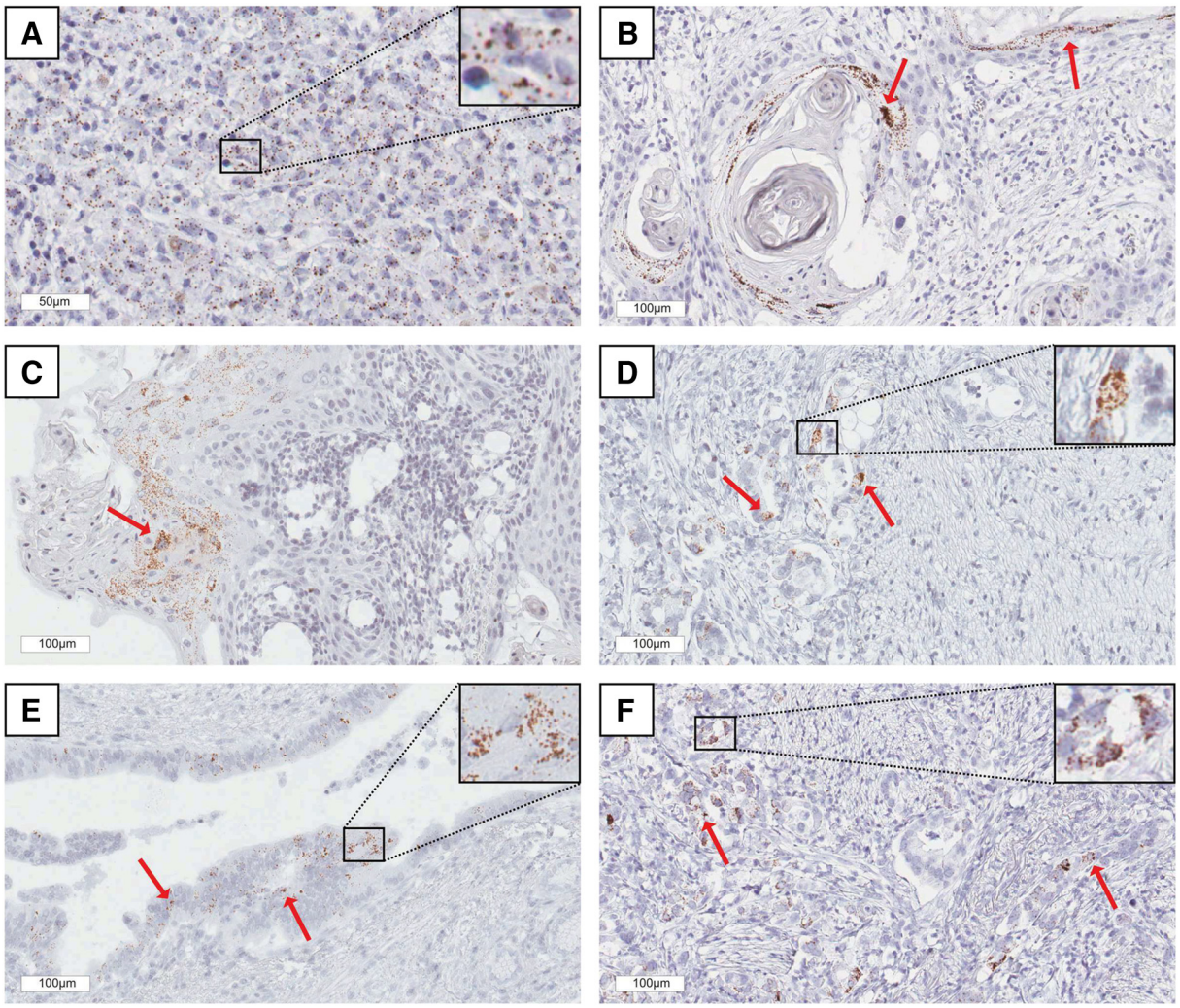

Figure 1. (A) Representative example of RNA ISH single-plex assay performed on FFPP section of pancreatic tumor metastasized in skin using the positive control probe *POLR2A.* Brown dots show expression of *POLR2A* using the RNAscope 2.5 HD Reagent Kit-BROWN. (Magnification x20). (B) Squamous cell carcinoma with keratinization. *SERPINB7* is expressed in the tumor cell layer beneath the keratin. In normal skin this layer would be compatible with stratum granulosum. Arrows are indicating tumor cells. (Magnification ×20). (C) Squamous cell carcinoma used as positive control. (Magnification ×8). (D) PDAC with expression of SERPINB7 in some tumor cells. Arrows are indicating tumor cells expressing SERPINB7. (Magnification ×20). (E) Arrows are indicating reactive pancreatic ducts which are embedded in pancreatic tumor tissue and express SERPINB7 (Magnification ×20). (F) Arrows are indicating some tumor cells expressing SERPINB7 (Magnification ×20).

Results

Patient Characteristics

All patients who underwent surgery for tumor resection and were treated with gem or gem/nab as adjuvant therapy in our institution between 2011 and 2017, were included in this study (n = 57) (Table 1). At the time of analysis (February 2018), 17 patients were still alive. Follow-up was available for all patients and the median follow-up was 19.80 months (range: 3.6–77.5 months). All patients received adjuvant chemotherapy: 46 received gemcitabine and 11 received gemcitabine plus nab-paclitaxel (gem/nab). In the gemcitabine group, there were 18 females (39.13%), 37 patients (80.43%) had lymph node metastases and two patients (4.35%) had distant metastases at initial diagnosis. In the gem/nab group, 3 patients (27.27%) were females, 9 patients (81.82%) had lymph node metastases and one patient (9.09%) had distant metastases at diagnosis. 17 patients (out of 57) (29.82%) had positive resection margins.

Considering the whole cohort, 65.2% of the patients (Confidence Interval (CI): 51.0% - 76.3%) survived at least one year. According to the chemotherapy treatment, the overall 1-year survival was 65.7% (CI: 49.6%-77.7%) in the gem group and 63.6% (CI 29.7%-84.5%) in the gem/nab group (Supplemental Figure 1). The median DFS was 9.4 months and 9.1 months (P = .142) and the median OS in censored patients was 15.7 months and 21.20 months (P = .279) in the gemcitabine and gem/nab group, respectively. Among the baseline characteristics depicted in Table 1, only early tumor stage (T1/T2) had an influence on DFS in the univariate (P = .01) and multivariate analysis (P = .033) (data not shown). Early tumor stage (T1/T2) was also correlated with OS in the univariate analysis (P = .01) but not in the multivariate analysis (P = .081). The presence of metastasis at diagnosis had a correlation on DFS and OS by using the log-rank test (P = .01) but not in the Cox regression model (P = .229 and P = .352,

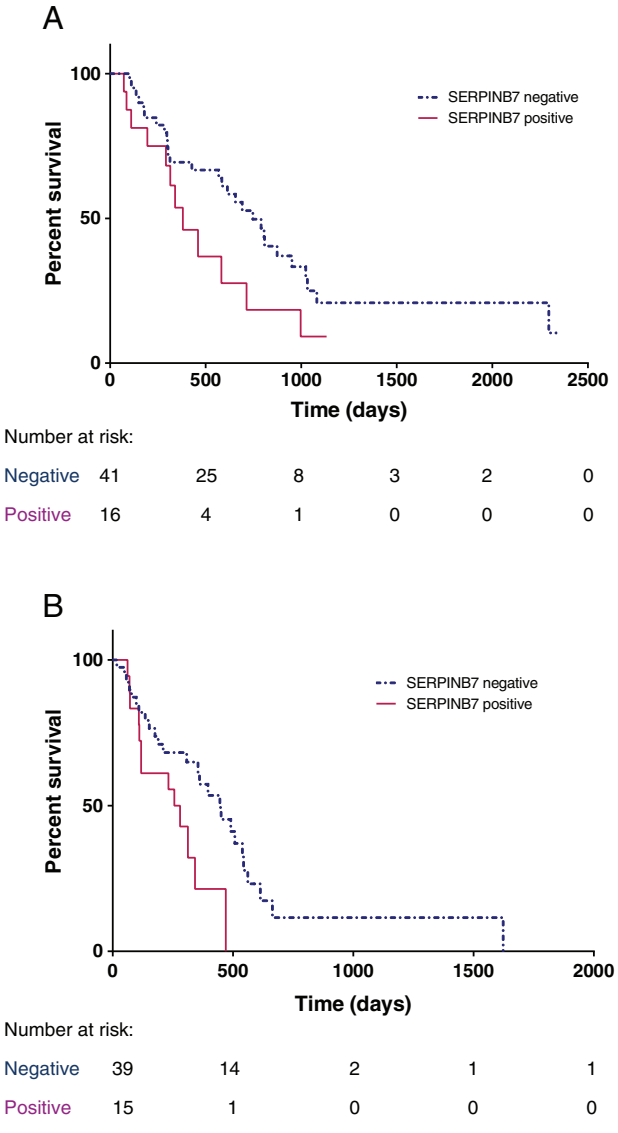

Figure 2. OS (A) and DFS (B) of the 57 patients according to the RNA expression of SERPINB7 and regardless of the therapy they received. Univariate analysis showed a correlation of SERPINB7 and OS (P = .01) but not with DFS (P = .844). The correlation between SERPINb7 and OS could not be confirmed in the multivariate analysis (Hazard ratio (HR): 2.22; 95% CI: 1.05–4.70, P = .107).

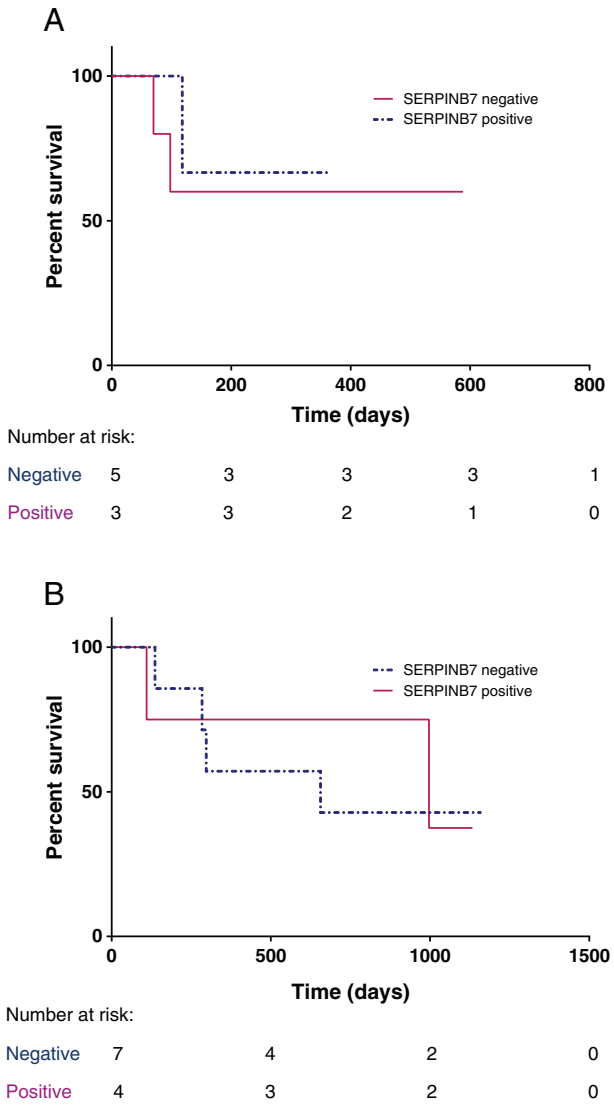

Figure 3. DFS (A) and OS (B) of patients that received gem/nab as adjuvant therapy. No significant difference was observed between DFS or OS and SERPINB7 expression (P = .719 and P = .745, respectively).

respectively) (data not shown). None of the other baseline characteristics was statistically significantly associated with DFS or OS.

RNA Expression of SERPINB7 is a Predictor of Poor Outcome in Patients With Pancreatic Cancer Treated With Gemcitabine

The RNA level of SERPINB7 was qualitatively assessed by RNA-ISH in tumor samples of 57 PDAC patients. Expression of DapB and the low-expressed housekeeping gene POLR2A (Figure 1*A*) were used as negative and positive control probes, respectively. In a first step, our custom-designed SERPINB7 RNA-ISH assay was tested on head and neck squamous cell carcinoma tissue samples, which was used as positive control based on the Human Protein Atlas [22,23]. As expected, SERPINB7 expression was detected in the stratum granulosom of these samples (Figure 1, *B* and *C*) suggesting that our assay is suitable for analyzing SERPINB7 RNA expression in FFPE tissues. In a next step, we analyzed SERPINB7 RNA expression in our cohort of 57 PDAC patients. 16 of these patients (28%) were positive for SERPINB7 RNA expression in tumor cells and/or in some reactive pancreatic ducts (Figure 1, *D*–*F*). Of note, RNA expression of SERPINB7 was not equally distributed throughout the tissue but it was present in small groups of tumor cells (Figure 1, *D* and *F*). Furthermore, statistical analysis revealed that expression of SERPINB7 did not correlate with any of the clinicopathological characteristics shown in Table 1 (Supplementary Table S1). SERPINB7-positive patients had a median DFS of 8.4 months (range: 2.0–15.2 months) and a median OS of 10.7 months (range: 2.3–36.5 months), while SERPINB7-negative patients had a median DFS of 10.3 months (range: 0.5–52.4 months) and a median OS of 21.2 (range: 3.3–75.4 months).

To investigate the prognostic significance of SERPINB7 RNA expression in PDAC patients, we first tested whether the expression of this gene correlated with DFS or OS in the whole cohort (n = 57). Univariate analysis revealed that expression of SERPINB7 correlated with OS (P = .01) (Figure 2*A*) but not with DFS (P = .844) (Figure 2*B*). However, expression of SERPINB7 did not reach statistical significance in the multivariate analysis (Hazard ratio (HR): 2.22; 95% CI: 1.05–4.70, P = .107). When patients were grouped according to the adjuvant treatment, we observed that there were no significant differences in DFS (P = .719) or OS (P = .745) between patients with or without SERPINB7 expression in the gem/nab group (Figure 3*A* and B). However, in the gem group, expression of SERPINB7 was associated with a shorter DFS (two-stage test, P = .01) (Figure 4*A*) and a shorter OS (log-rank test P = .002) (Figure 4*B*). Cox-regression analysis confirmed the independent predictive value of the expression of SERPINB7 on OS (P = .006, HR: 3.47; 95% CI: 1.49–8.09) in the gem group.

Protein Expression of SERPINB7 Does Not Have Any Prognostic or Predictive Value in Pancreatic Cancer

To test if SERPINB7 RNA expression also correlates with SERPINB7 protein expression, we performed immunohistochemical analyses on 56 out of 57 tumor samples from PDAC patients. Like for SERPINB7 RNA expression, SERPINB7 protein expression was first analyzed in positive controls (head and neck squamous cell carcinoma tissue samples). Comparable with RNA expression, we detected high SERPINB7 protein expression in the stratum granulosom of the squamous cell carcinoma samples. In addition, focal low SERPINB7 expression was seen in the stratum granulosom of skin. Representative examples of positive and negative controls are presented in Figure 5, *A* and *B*. These data indicate that our IHC assay is suitable for detecting SERPINB7 expression in FFPE samples. Thus, we determined SERPINB7 protein levels in our PDAC patient cohort and detected SERPINB7 in the cytoplasm of the island of Langerhans in 55 out of 56 tumor samples (Figure 5*C*). For the evaluation of the protein level of SERPINB7, samples were considered as positive if the protein level was detected in tumor cells or reactive ducts. Thirty-eight patients (67.88%) were SERPINB7-positive (Figure 5D). Furthermore, no association was found between SERPINB7-RNA and protein expression. According to the SERPINB7 protein expression, no differences were found in DFS or OS neither in the gem nor in the gem/nab group (Figure 6, *A*–*D*).

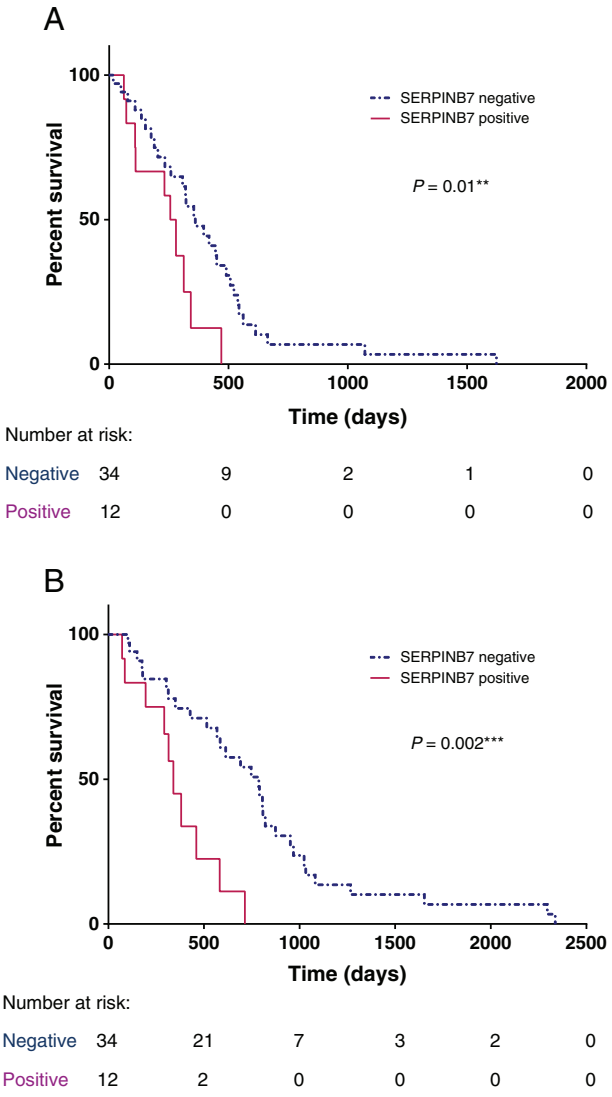

Figure 4. DFS (A) and OS (B) of patients that received gem as adjuvant therapy. Univariate analysis showed that SERPINB7 was associated with a poor DFS (two-stage test, P = .01) and poor OS (log-rank test P = .002). Multivariate analysis confirmed the independent predictive value of the expression of SERPINB7 on OS (P = .006, HR: 3.47; 95% CI: 1.49–8.09) in the gem group.

Discussion

There are growing appeals for encouraging research in pancreatic cancer so that mortality rate predictions do not come true. In an effort to contribute to this goal, our group is focused on the identification of RNA

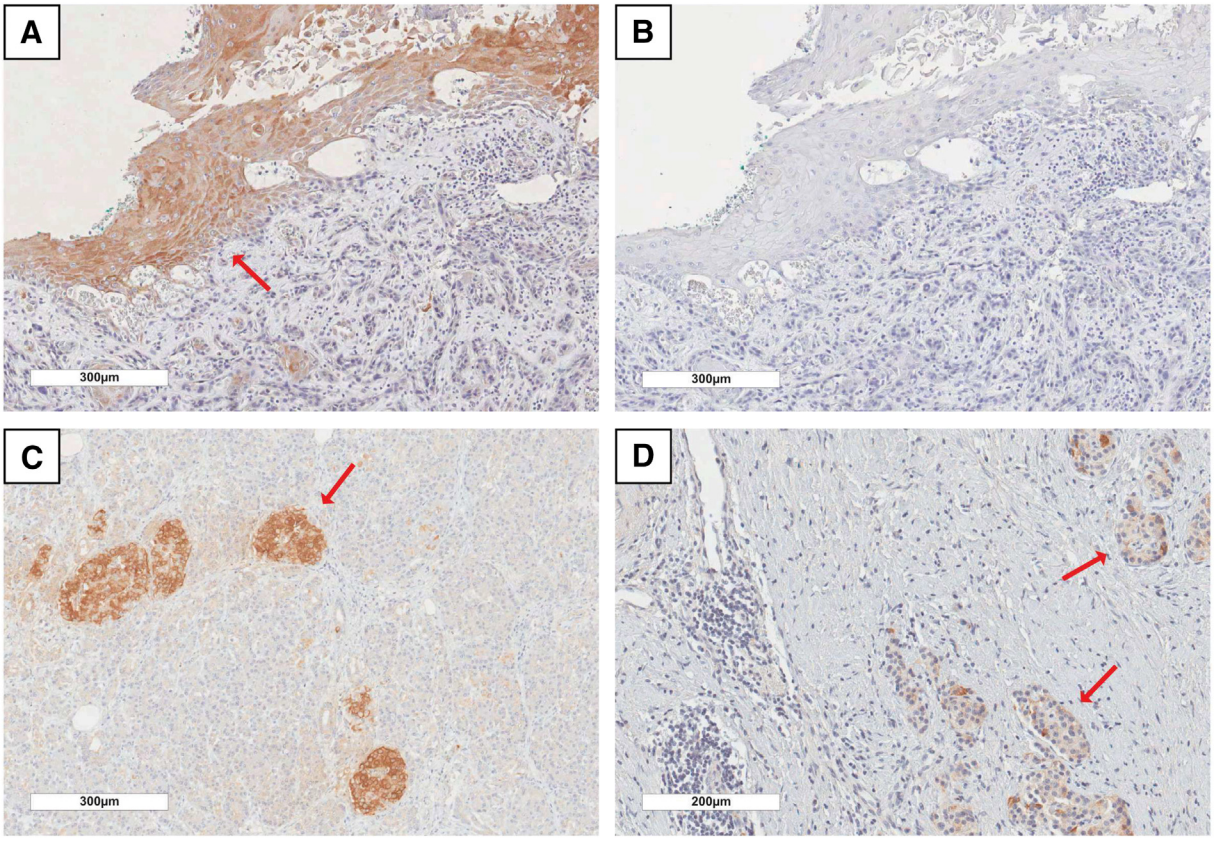

Figure 5. Squamous cell carcinoma used as positive (A) and negative (B) control, respectively, for immunohistochemistry. (Magnification 8×). (C) Representative example of high expression of SERPINB7 in the islets of Langerhans (arrow) (Magnification 10×). (D) Representative example of SERPINB7 expression in tumor cells (arrows) (Magnification 10×).

biomarkers that may help to identify patients that are more likely to benefit from specific therapies. To the best of our knowledge, we report for the first time that expression of SERPINB7 is a poor predictor of DFS and OS for the adjuvant treatment of pancreatic cancer patients with gemcitabine. So far, only little is known about SERPINB7. High expression of SERPINB7 was originally identified in human mesangial cells, which is the reason why this gene was initially termed megsin [24]. There is strong evidence indicating that specific loss-of-function mutations in this gene are responsible for a common Asian type of palmoplantar keratosis called Nagashima-type palmoplantar keratoderma [25,26] and it was shown that expression of SERPINB7 is up-regulated in IgA- and diabetic nephropathy [24,27]. Until now, little research has been conducted on the functional role of this protein. The few cutting-edge investigations in this field have proven that SERPINB7 overexpression in transgenic mice and rats led primarily to nephritis characterized by hyperproliferation in mesangial cells and matrix accumulation with augmented immune complex deposition [28,29]. Moreover, it was shown that SERPINB7 overexpression in rats led to development of serpinopathy, which is characterized by the accumulation of SERPINB7 protein in the exocrine and endocrine cells of the pancreas and by apoptosis of β cells [30]. Plasmin was identified as a biological substrate of SERPINB7 and in vitro studies revealed that overexpression of this protein in rat cells was associated with enhanced expression of some cytokines such as TNF-alpha and secretion of collagen type IV [31–33]. Regarding the functional role of SERPINs in cancer, Valiente et al. reported that some anti-plasmin-SERPINs expressed in the brain promoted cancer cell survival and metastasis [34]. By the use of the latest RNA ISH method, we were able to detect mRNA expression of SERPINB7 in FFPE tumor samples from patients with pancreatic cancer. We found that SERPINB7 expression varies between tumor samples suggesting that expression of this gene is deregulated in pancreatic cancer. Thus, the finding presented herein and our previous results [16] together with this body of evidence confirm that SERPINB7 is aberrantly expressed in pancreatic cancer and let us hypothesize that it might play a role in enabling metastasis of pancreatic tumor cells, as it is the case of brain cancer [34]. Moreover, considering the primary site of expression of SERPINB7 under normal conditions (stratum granulosum of the skin), it is noteworthy to mention that this layer is of crucial importance for the formation of the hydrophobic barrier of the skin [35]. Therefore, if SERPINB7 is somehow involved in the formation of this barrier in the skin, it is tempting to speculate that this also happens in pancreatic cancer, which might explain why patients expressing SERPINB7 are resistant to the hydrophilic gemcitabine chemotherapy.

We also analyzed SERPINB7 protein expression and compared these data with mRNA expression data and clinicopathological characteristics of pancreatic cancer patients. Interestingly, the protein level of SERPINB7 was higher than the RNA level (Figures 1 and 5) and it did not show any predictive or prognostic value. Although this discrepancy between the RNA and protein level is already known and widely accepted in the scientific community, we can only hypothesize about the reasons for this difference [36]. SERPINs are 'kamikaze' proteins that are irreversibly inactivated by binding to their substrates and in some pathological conditions, they polymerize and form aberrant intracellular protein clusters [37]. This pathology is called

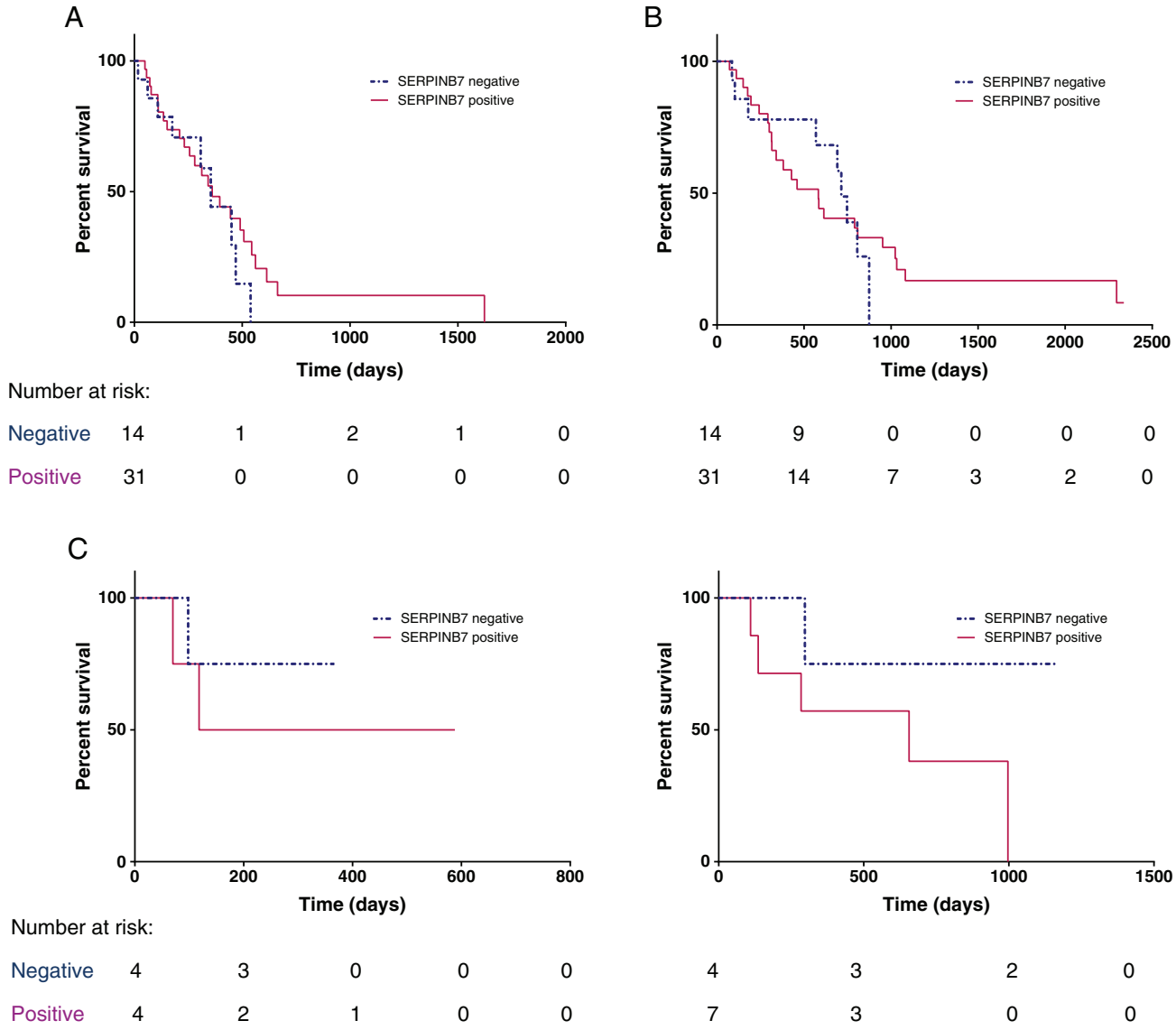

Figure 6. Kaplan–Meier curves of DFS and OS in dependence of protein expression level of SERPINB7 in the gem group (A and B) and gem/nab (C and D) group. No significant difference was found between protein expression of SERPINB7 and OS or DFS.

serpinopathy and it was already reported in vivo in the pancreas and kidney of rodents overexpressing SERPINB7 [30]. Thus, one possible explanation for the higher protein level of SERPINB7 might be the presence of such intracellular clusters [38]. Another reason might be of methodological nature. For instance, immunostaining was performed by using a polyclonal antibody, which might lead to an enhanced signal in comparison to the RNA level detected by RNA ISH. It is important to mention that notwithstanding the cause of the discrepancy between the RNA and protein levels, we demonstrated herein that the detection of RNA of this gene in archival FFPE material correlated with a poor DFS and OS in patients with pancreatic cancer treated with gemcitabine as adjuvant therapy.

Limitations and Conclusions

Despite the promising results presented herein, this work has some limitations mostly due to the intrinsic characteristics of the study. It is a retrospective study and as such, the number and distribution of patients in each arm was limited. Furthermore, this study did not include patients with resected PDAC treated with gemcitabine plus capecitabine or mFOLFIRINOX, which became new options for the adjuvant treatment of pancreatic cancer after this study was completed. The ESPAC-4 and PRODIGE 24 clinical studies have recently shown that this two regimens prolonged survival in a certain subset of patients with resected pancreatic cancer in comparison to patients treated with gemcitabine alone [39,40]. In addition, the results of the APACT study (NCT01964430) aiming to test the efficacy of gemcitabine plus nab-paclitaxel as adjuvant chemotherapy for pancreatic cancer are pending. Thus, it is still uncertain whether these combined treatments that are now being recommended as adjuvant therapy will become the standard of care.

Another issue that might be seen as a limitation is the fact that expression of SERPINB7 did not correlate with the R margin status. Nonetheless, it is intriguing to note that although the whole tumor (R0) and sometimes even the whole pancreas are removed, the 5- and 10-year survival rates for this disease are 18% and 13%, respectively [41]. Evidence strongly suggest that there are indeed micrometastases

at the time of diagnosis, even if metastatic lesions are not detectable via conventional methods such as computed tomography scans [42–45]. Therefore, and according to our research on the expression of SERPINB7 (published and unpublished data), we can affirm that: (1) SERPINB7-RNA is expressed in hepatic metastatic lesions, (2) SERPINB7-RNA is expressed in primary pancreatic tumor, (3) SERPINB7-RNA expression seems to increase with cancer progression (unpublished data) and that (4) its expression seems to correlate with a poor outcome. Thus, we hypothesize that tumor cells that express SERPINB7 have the ability to metastasize (at least) the liver and that the expression of SERPINB7 in the resected primary pancreatic cancer would be an indicator of the presence of hepatic micrometastatic lesions. This hypothesis would explain why SERPINB7 expression correlates with a poor prognosis even if the apparent whole tumor is removed.

In conclusion, we demonstrated herein that expression of SERPINB7 at the RNA level might represent the first biomarker that enables stratification of patients with pancreatic cancer. These findings may be of clinical relevance because if RNA-SERPINB7 is detected in the tumor tissue, patients should receive an hydrophobic chemotherapeutic agent as adjuvant therapy rather than gemcitabine alone. Although our patient cohort was relatively small and our data need to be validated in additional studies, we are convinced that the results of this study will bring the scientific community one step closer to the devising of an individualized therapy to treat the life-threatening disease pancreatic cancer.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.08.019.

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Conflict of Interest

The authors declare no conflict of interest.

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