Plasma seprase and DPP4 levels as markers of disease and prognosis in cancer

Mazyar Javidroozi^{a,b,*}, Stanley Zucker^{a,c} and Wen-Tien Chen^a

^aDepartment of Medicine, Stony Brook University, Stony Brook, NY, USA

^bDepartment of Anesthesiology, Englewood Hospital and Medical Center, Englewood, NJ, USA

^cDepartments of Research and Medicine, Veterans Affairs Medical Center, Northport, NY, USA

Abstract. Seprase (fibroblast activation protein α) has been examined as an invasion biomarker for various types of solid tumors. We studied whether plasma levels of seprase and homologous protease, DPP4 in cancer might serve as tumor biomarkers. We developed sensitive and specific Enzyme-Linked Immunosorbent Assays (ELISAs) to measure these proteases. In 747 plasma samples (from 139 healthy volunteers and 561 cancer patients), mean seprase and DPP4 levels were 0.51 ± 0.30 and $4.65 \pm 6.37 \mu\text{g/mL}$, respectively, and they were correlated with each other ($R^2 = 0.382$). Plasma DPP4 and seprase levels were significantly lower in cancer patients compared with healthy subjects (4.38 versus $5.65 \mu\text{g/mL}$, $p < 0.001$ for DPP4; 0.46 versus $0.66 \mu\text{g/mL}$, $p < 0.001$ for seprase). Higher DPP4 was associated with better survival in all cancers combined ($n = 346$) as well as in head and neck malignancies ($n = 38$). Higher seprase was associated with better survival in all non-metastatic cancers combined ($n = 151$) as well as head and neck malignancies, but worse survival in colorectal cancers ($n = 47$). This study demonstrates that in contrast to the high expression in solid tumors, plasma concentrations of seprase and DPP4 are reduced and correlate inversely with survival in most types of cancer, suggesting that these circulating proteases represent useful tumor markers.

Keywords: Seprase, fibroblast activation protein α (FAP- α), DPP4, prognosis, cancer, tumor marker

1. Introduction

Human seprase (fibroblast activation protein α [FAP α]; Gene ID: 2191, accession number: NP_004451; protein accession number: Q12884, gi: 160113236) is a 170 kDa homodimeric glycoprotein consisting of two 97 kDa subunits. Seprase and the other well-known member of the dipeptidyl prolyl peptidase (DPP) family, DPP4, are membrane-bound serine proteases [3,22,23,34]. Seprase and DPP4 share 50% overall amino acid sequence and 70% amino acid sequence homology in the catalytic domain. The corresponding genes share 63% identity and they are both located on chromosome 2 with less than 100 kb distance

from each other, suggestive of a gene duplication event. DPP4 and seprase are known to form a hetero-oligomer in a proteolytic complex, which plays a role in the invasion of cells in collagenous matrices [16,17]. Their coupled expression in neoplastic cells has been reported [2]. Seprase is an integral membrane protein and it lacks a secretory signal peptide. Seprase is shed from cells as soluble forms *in vitro*; further activation of its proteolytic activity through metalloprotease-mediated truncation has been described [4]. Soluble seprase has also been identified in blood, with the initial description of its role in coagulation as antiplasmin-cleaving enzyme (APCE) [30]. Other suggested natural substrates of seprase include neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY [27].

An increasing body of evidence suggests that expression of the membrane-bound seprase in various solid tumors is associated with tumor growth and invasion and poor prognosis [5,7,13,21,23,25,28,29,35,39,47],

*Corresponding author: Mazyar Javidroozi, M.D., Ph.D., Department of Anesthesiology, Englewood Hospital and Medical Center, 350 Engle Street, Englewood, NJ 07631, USA. Tel.: +1 201 894 3917; Fax: +1 201 894 0585; E-mail: mazyarjr@yahoo.com.

making seprase an attractive subject for study as a tumor biomarker, as an antigen for tumor imaging, and as a potential therapeutic target [45]. Welt et al. [42] initially described the possible diagnostic and therapeutic use of humanized monoclonal antibodies (F19) directed against FAP α /seprase. Although preclinical studies on F19 immunoconjugates suggested that seprase could serve as a therapeutic target [36], the humanized F19 monoclonal antibody exhibited limited therapeutic activity in a Phase I clinical trial [40]. Likewise, administration of a small-molecule inhibitor of seprase, Val-boro-Pro (Talabostat), resulted in minimal clinical activity in pre-treated patients with metastatic colorectal cancer; however, incomplete inhibition of seprase enzymatic activity in the peripheral blood was observed in this study [33]. Additionally, it has recently been reported that catalytically-inactive seprase (as a result of inhibitors or mutations) can still promote tumor growth and invasion, suggestive of other, non-enzymatic pathways [24,41].

In spite of considerable research focusing on tumor-associated seprase, the clinical utility of measuring plasma levels of seprase in patients with cancer has not been investigated. The aims of the research described herein were to compare seprase and DPP4 levels in the plasma of patients with cancer and healthy individuals, and to evaluate their role as markers of disease and potential prognostic factors.

2. Materials and methods

2.1. Human subjects

Plasma samples used in this study were procured from consenting adult volunteers under Institutional Review Board-approved protocols. Participants consisted of (self-declared) healthy individuals and patients newly diagnosed with various malignancies at Stony Brook University Cancer Center (Stony Brook, NY) and Veterans Affairs Medical Center (Northport, NY). Data on presence of other comorbidities were not available. Venous blood for this study was collected shortly after diagnosis of cancer and prior to surgery and chemo/radiotherapy in the out-patient clinic. A limited number of subjects had subsequent blood-draw(s) following surgery and chemo/radiotherapy (collected during hospital stay or after discharge). In each case, approximately 5 mL blood was drawn into BD green-capped Vacutainer[®] tubes containing 76 IU lithium heparin, placed on ice and immediately transferred to

the laboratory. Samples were centrifuged at 2,500 g for 10 minutes; plasma was carefully removed, aliquoted and stored at -80°C until used. The duration of patient follow-up spanned from the time of cancer diagnosis (which was usually within a few days of obtaining the blood sample) to the last visit or time of death.

2.2. Production and purification of recombinant soluble seprase and DPP4

HEK293 cells were transfected with a modified pCEP4 vector harboring the seprase sequence without the transmembrane domain and with an N-terminus mouse Ig κ secretion signal and a C-terminus V5-His tag; cells were selected initially using G418 and Hygromycin, as described [4]. Drug-resistant clones were further screened for secretion of recombinant soluble seprase (r-seprase) into medium by an antibody-capture enzymatic assay, serially testing for DPP and gelatinase activities, as described [4]. The clones with highest expression of r-seprase were identified and cultured in serum-free medium (SFM4HEK, Hyclone) to produce high levels of r-seprase. Cell-conditioned medium was preserved by addition of EDTA to a final concentration of 5 mM to suppress further proteolysis and degradation of seprase by metalloproteinases.

Culture medium was cleared and proteins were precipitated by ammonium sulfate (80%), resuspended in PBS, and dialyzed against buffer, and r-seprase was purified relying on its C-terminus V5-His tag, using a charged His-Bind Resin column (Novagen), eluted with 1M imidazole. Eluted protein was concentrated using Ultrafree-15 Centrifugal Filter Devices (Millipore) and fractionated using a Superdex 200 Prep grade gel filtration column (Pharmacia Biotech) as previously described [4]. Purity of recombinant protein was verified using SDS-PAGE and the protein was confirmed to be r-seprase using immuno-blotting and antibody-capture enzymatic assays for DPP and gelatinase activities. A similar procedure was used to produce and purify recombinant DPP4 (rDPP4), using the same modified pCEP4 vector (Invitrogen) harboring the DPP4 extracellular domains as well as the secretion signal of mouse Ig kappa-chain at N-terminus and the V5-His fusion tag at C-terminus.

2.3. Development of seprase and DPP4 ELISAs

Enzyme-linked immunosorbent assays (ELISA) to measure seprase and DPP4 in plasma were developed using previously generated monoclonal antibod-

ies against seprase and DPP4. Various seprase monoclonal antibody combinations were tested to determine the best pair to yield a sensitive and specific assay, and monoclonal antibodies D8 and D28 were chosen for seprase ELISA. These monoclonal class-2a IgGs were originally derived from the fusion of rat myeloma Y3 cells and the spleen cells of Sprague-Dawley rats that were immunized with partially purified seprase derived from human placenta as previously reported [19,31,37]. In addition to recognizing purified protein using immune-precipitation, D8 and D28 antibodies specifically bind non-reduced human seprase homo-dimer (isolated from LOX human metastatic melanoma cells) at 170-kDa and dissociated 97-kDa monomers, as resolved by Western immunoblotting analysis. However, these antibodies did not recognize the intracellular small form of seprase [18], nor did these antibodies react with the extracellular active truncated forms of seprase [4]. Microtiter U bottom 96-well plates (Dynex Technologies) were coated with rat anti-human-seprase (monoclonal antibody D28) and blocked with 5% bovine serum albumin (BSA) in PBS. Samples to be tested were thawed and mixed, and serially diluted to 0.1% in ELISA assay buffer (1% BSA, 10 mM EDTA, 0.05% Tween 20 in PBS) and incubated with Digoxigenin-conjugated rat anti-human seprase (monoclonal antibody D8) diluted in ELISA assay buffer. Sample-antibody mixtures were added to the antibody-coated 96-well plates and incubated at room temperature on a shaker. Following 3 washes with PBS containing 0.05% Tween 20, anti-Digoxigenin-POD poly Fab fragment (Roche) diluted in ELISA assay buffer was added to the wells and incubated at ambient temperatures on a shaker. Following the final round of washing with PBS containing 0.05% Tween 20, TMB liquid substrate for ELISA (Sigma) was added to the wells and color reaction was monitored until adequate color development, at which point, the reaction was stopped by adding HCl to the wells. Plates were read at 450 and 570 nm by a Microplate Spectrophotometer System (Molecular Devices), and the 570 nm reading values were subtracted from the 450 nm readings to obtain the final value for each well. Serial dilutions of the purified r-seprase in ELISA assay buffer was used as standard. Each sample was tested in triplicate.

A similar ELISA was developed to measure DPP4 in plasma using the monoclonal anti-DPP4 antibody pair of E26 (coating the plate) and E3 (Digoxigenin-conjugated). These monoclonal class-2a IgGs were produced by a hybridoma cell line derived from fu-

sion of rat myeloma Y3 cells and spleen cells of a Sprague-Dawley rat immunized against purified human placental DPP4 as previously described [14,15,19,31]. In addition to recognizing purified protein using immune-precipitation, E26 and E3 identify non-reduced human DPP4 homo-dimer (isolated from LOX human metastatic melanoma cells) at 220-kDa, as resolved by Western immunoblotting analysis. Serial dilutions of purified rDPP4 in ELISA assay buffer were used as standard for these wells.

Results of each assay were expected to meet the following acceptance criteria: Coefficient of Variation (CV) less than 10%; measurement within the linear standard range; and R^2 of the standard curve regression line at least 0.95. CV was used as a normalized measure of dispersion of the individual readings in triplicate.

The performance of the developed seprase and DPP4 ELISAs was verified. Using serial dilutions of recombinant proteins, the lowest detection level of seprase and DPP4 assays were estimated to be 60 pg/mL and 600 pg/mL, respectively. Given these observations and linear range of the measurements, the seprase and DPP4 standards of the assays were prepared to be in the range of 0.06-1.9 μ g/mL for seprase and 0.6-19.2 μ g/mL for DPP4 (Fig. 1A and 1B). Any readings not falling in these ranges were repeated at other adjusted dilutions.

Furthermore, the assays were specific for the target protein as indicated by the following: 1) Each assay showed no cross-reactivity with the opposite recombinant human protein (i.e. rDPP4 was indistinguishable from the negative control in the seprase ELISA, and vice versa.); 2) the assays showed no cross-reactivity with bovine seprase and DPP4; 3) the assay showed no cross-reactivity with other proteins including non specific immunoglobulins from rat, bovine, or human sources, as well as human plasma samples depleted of the target proteins (seprase or DPP4) using antibody coated beads.

Following addition of 1.52 μ g r-seprase to plasma samples containing 0.45 μ g seprase as previously measured by ELISA, the total measured seprase increased to 2.20 μ g (i.e. 111% of the expected total) in the total volume of 1 mL, while the DPP4 level measured by ELISA changed only 0.6%. Similarly, following addition of 4.8 μ g rDPP4 to plasma samples containing 6.56 μ g DPP4, the total measured DPP4 increased to 9.05 μ g (i.e. 80% of the expected total) in the total volume of 1 mL, while the seprase measurement changed 3.8% (Fig. 1C).

Finally, we validate that antibodies used in ELISAs did in fact recognize the enzymatic activities of target

Fig. 1. Assay performance. A) Assay reading (in terms of optical density [OD] at 450 nm minus 570 nm) using serial dilutions of seprase. Error bars represent standard deviation. B) Assay reading using serial dilutions of DPP4. Error bars represent standard deviation. C) Spiking experiments: the baseline levels of DPP4 and seprase in human plasma samples were measured using ELISA ("Baseline") and known amounts of r-DPP4 or r-seprase added to plasma samples ("Spiked"). The new levels of DPP4 and seprase following addition of recombinant proteins were measured using ELISA ("Final"). Values are presented by the height of the bars. D) Intra- and inter-assay coefficient of variation (CV) for DPP4 and seprase ELISA. Error bars represent standard deviation. E) Scatter plot of plasma DPP4 versus seprase levels measured in the study population. $N = 692$; Eight data points falling out of axis ranges were omitted.

proteins. DPP enzymatic assays were performed on 4 randomly-selected plasma samples, using D28 mAb (to capture and assess seprase activity) or E19 mAb (to capture and assess DPP4 activity) as previously described [4]. The DPP activity of the samples were found to correlate with the ELISA antigenic measurements (Spearman's correlation coefficient 0.660, $p = 0.020$ for seprase and 0.691, $p = 0.013$ for DPP4).

Immuno-assays were then used to measure seprase and DPP4 in clinical samples. Overall intra-assay CVs for seprase and DPP4 ELISA were below 5% (Fig. 1D). The mean inter-assay CV for seprase and DPP4 ELISA were below 15% in independently performed assays (Fig. 1D).

2.4. Statistical analysis

DPP4 and seprase concentration data were verified by Kolmogorov-Smirnov and Shapiro-Wilk tests of normality to follow a normal distribution; logarithmic transformation was applied when necessary. Data are expressed as mean $\pm$ standard deviation, or median (followed by interquartile range [IQR]) unless otherwise specified. Student's t-test was used to compare the DPP4 and seprase level between male and female subjects as well as healthy participants and cancer patients. Similarly, ANOVA with Bonferroni *post hoc* test was used to compare the DPP4 and seprase levels among different cancer types, and different tumor size

(T), regional lymph node status (N), and distant metastasis (M) used in TNM staging system as well as the overall TNM staging itself. If the normality assumption was not met, non-parametric variants of the above mentioned tests (i.e. Mann-Whitney U-test and Kruskal-Wallis H-test) were used as needed. Simple bivariate Pearson or Spearman's Rho tests were used as appropriate to study the correlation between DPP4, seprase and continuous variables following visual examination of scatter plots to rule out outliers and specific patterns. The overall effect of DPP4 and seprase levels on survival was studied using Cox regression. Kaplan-Meier analysis with Log Rank test was also used to evaluate the association between DPP4 and seprase levels and overall survival, comparing groups created by cut-off points based on median DPP4 and seprase levels (i.e. patients with seprase levels above median versus below median). Analyses were performed on various cancer cases combined as well as subgroups of common cancer types. All analyses were done using SPSS software (Version 16.0; Chicago, IL) and results were considered to be statistically significant if the p value was less than 0.05. Assuming an alpha of 0.05, 17 samples per arm was sufficient to detect a difference of one standard deviation in seprase levels between the compared arms using unpaired t-test with power of 80%. Therefore, subgroup comparisons were focused on cancer type with at least 17 cases per arm.

3. Results

3.1. Plasma seprase and DPP4 levels of healthy individuals and cancer patients

A total of 747 plasma samples were obtained from 700 subjects: 656 subjects had one sample and 44 had two or more samples obtained at other times (due to participation in other studies). Unless otherwise stated, the analyses presented in this manuscript are based on 700 plasma samples obtained on first visits. The distribution of the samples and the patients' demographics are presented in Table 1.

In the 747 plasma samples studied (cancer and healthy combined), mean seprase and DPP4 levels were 0.51 ± 0.30 and $4.65 \pm 6.37 \mu\text{g/mL}$, respectively. Two gynecologic cancer patients had extremely high DPP4 levels in excess of $100 \mu\text{g/mL}$, as verified by multiple independent measurements; these two cases were excluded from parametric analyses (mean DPP4 level after exclusion of these 2 cases was $4.33 \pm 2.24 \mu\text{g/mL}$;

mean seprase level was unchanged). The DPP4 and seprase levels were correlated with each other, but several outliers were present as well ($R^2 = 0.382$, $p < 0.001$; Fig. 1E). Levels of DPP4 and seprase were slightly higher in female subjects, although it reached statistical significance only in the case of DPP4, and they were inversely associated with age (Pearson correlation -0.255 , $p < 0.001$ for DPP4 and Pearson correlation -0.249 , $p < 0.001$ for seprase).

In healthy subjects, there was no significant association between age and DPP4/seprase levels. Seprase levels were significantly higher in male versus female subjects (0.76 ± 0.18 versus $0.65 \pm 0.18 \mu\text{g/mL}$, respectively, $p = 0.015$). Similarly, DPP4 levels were higher in healthy male versus female subjects, ($6.31 \pm 1.21 \mu\text{g/mL}$ versus $5.65 \pm 1.48 \mu\text{g/mL}$, respectively, $p = 0.039$).

Plasma seprase and DPP4 levels were significantly lower in cancer patients compared with healthy subjects ($3.95 \pm 2.29 \mu\text{g/mL}$ versus $5.69 \pm 1.47 \mu\text{g/mL}$, respectively, $p < 0.001$ for DPP4; 0.47 ± 0.30 versus $0.66 \pm 0.19 \mu\text{g/mL}$, respectively, $p < 0.001$ for seprase). The cancer patients were significantly older than the healthy subjects (mean age 64.79 ± 12.13 versus 43.12 ± 14.16 years old, $p < 0.001$). To further evaluate the potential effect of age on DPP4/seprase levels in cancer patients and healthy subjects, the comparison was repeated between 87 healthy subjects and 109 age-matched cancer patients (mean age 50.48 ± 9.71 years in healthy subjects versus 51.89 ± 8.65 years in cancer patients, $p = 0.285$). The gender distribution in the subgroups was similar (83.8% females in healthy subjects versus 76.9% females in cancer patients subgroup, $p = 0.300$). Plasma seprase and DPP4 levels were found to be significantly lower in cancer patients compared with healthy subjects in the matched subgroup analysis ($3.78 \pm 1.65 \mu\text{g/mL}$ versus $5.74 \pm 1.44 \mu\text{g/mL}$, respectively, $p < 0.001$ for DPP4; 0.45 ± 0.25 versus $0.67 \pm 0.19 \mu\text{g/mL}$, respectively, $p < 0.001$ for seprase).

In analysis of the data based on cancer type, seprase levels in normal subjects were significantly higher compared with patients with gynecologic cancer ($p < 0.001$), hematologic cancer ($p < 0.001$), head and neck cancer ($p < 0.001$), lung cancer ($p < 0.001$), upper gastrointestinal cancer (GI) cancer ($p < 0.001$), kidney and bladder cancer ($p = 0.018$), and colorectal cancer ($p = 0.030$) (Table 1). DPP4 levels in healthy volunteers were significantly higher than patients with gynecologic cancer ($p < 0.001$), hematologic cancer ($p < 0.001$), head and neck cancer ($p < 0.001$), lung cancer ($p < 0.001$), colorectal cancer ($p < 0.001$), and upper GI cancer ($p < 0.001$).

Table 1

Patient characteristics and plasma levels of DPP4 and seprase. Values are expressed as mean $\pm$ SD, except for tumor stage, which is expressed in median (interquartile range [IQR])

Subject category (n)	Male (%)	Age (years)	Metastatic cases (%)	Tumor stage	DPP4 ($\mu\text{g/mL}$)	Seprase ($\mu\text{g/mL}$)
Healthy (139)	18%	43 $\pm$ 14	–	–	5.69 $\pm$ 1.47	0.66 $\pm$ 0.19
Breast cancer (56)	0%	52 $\pm$ 13	17.8%	2 (2)	4.98 $\pm$ 1.94	0.60 $\pm$ 0.21
Hematological cancer (28)	33%	63 $\pm$ 10	57.1%	4 (2)*	3.90 $\pm$ 2.46 [§]	0.39 $\pm$ 0.31 [§]
Head and Neck cancer (55)	80%	67 $\pm$ 12	66.7%	4 (0)	3.87 $\pm$ 1.50 [§]	0.45 $\pm$ 0.22 [§]
Colorectal cancer (88)	52%	63 $\pm$ 12	69.8%	4 (0)	3.87 $\pm$ 1.53 [§]	0.55 $\pm$ 0.28 [§]
Lung cancer (100)	44%	65 $\pm$ 11	71.4%	4 (1)	3.71 $\pm$ 1.64 [§]	0.45 $\pm$ 0.47 [§]
Prostate cancer (45)	100%	75 $\pm$ 8	86.7%	4 (0)	5.70 $\pm$ 4.41	0.54 $\pm$ 0.20
Gynecologic cancer (100)	0%	63 $\pm$ 12	20.2%	3 (1)	5.07 $\pm$ 15.99 ^{†§}	0.33 $\pm$ 0.20 [§]
Upper gastrointestinal, Liver, and Pancreas cancer (27)	33%	72 $\pm$ 9	91.7%	4 (0)	4.02 $\pm$ 2.47 [§]	0.47 $\pm$ 0.23 [§]
Other/unknown cancer (62) [‡]	36%	69 $\pm$ 9	Unknown	Unknown	4.51 $\pm$ 2.39	0.54 $\pm$ 0.29
Total (700)	27%	59 $\pm$ 16	52.4%	4 (1)	4.65 $\pm$ 6.37	0.51 $\pm$ 0.30

*Only lymphomas; [†]Gynecologic malignancies included 2 cases of Clear Cell Adenocarcinoma with DPP4 levels of 109.66 and 121.99 $\mu\text{g/mL}$ based on repeated measurements. Mean DPP4 of gynecologic cancer cases after exclusion of these two cases was 2.81 $\pm$ 1.44, but mean seprase level stayed unchanged; [‡]Other/Unknown cancers included 16 urologic (other than prostate) cancers, 5 skin cancers, 1 brain cancer, 13 metastatic/secondary tumors of unknown origin, and 27 cases flagged as “cancer” but with insufficient clinical data available to determine the cancer type; [§] $p < 0.05$ compared with healthy subjects.

3.2. Correlation of plasma seprase and DPP4 levels with cancer stage

DPP4 levels in cancer patients of all types with ($n = 166$) and without metastasis ($n = 151$) were 4.26 ± 2.99 and 3.68 ± 1.92 $\mu\text{g/mL}$, respectively ($p = 0.101$). Seprase levels in cancer patients with and without metastasis were 0.50 ± 0.41 and 0.43 ± 0.22 $\mu\text{g/mL}$, respectively ($p = 0.111$). There was no significant difference in seprase or DPP4 levels among cancer patients based on tumor size or lymph node involvement; however, DPP4 level was significantly lower in patients with T3 compared with T1 tumors ($p < 0.001$).

When the overall TNM staging of the cancers was considered, DPP4 levels were significantly lower in stage III cancers compared with stages I ($p < 0.001$), II ($p < 0.001$) and IV ($p < 0.001$) cancer (Fig. 2A). Seprase levels were significantly lower in patients with stage III compared with stages II ($p = 0.017$) and IV ($p = 0.002$) cancer (Fig. 2B).

3.3. Temporal changes in plasma seprase and DPP4 measurements following treatment

In 44 cancer patients, a second blood sample was taken after patients underwent surgery (often during the course of chemotherapy or radiation therapy). The median time elapsed between 1st and 2nd blood sampling was 7 (IQR = 5) days. DPP4 and seprase measurements were highly correlated between the first and second specimens ($R^2 = 0.684$, $p < 0.001$ for DPP4 [Fig. 3A], and $R^2 = 0.332$, $p < 0.001$ for seprase

[Fig. 3B]). There was no correlation between the time elapsed from the 1st to 2nd blood samples and the difference in the 1st and 2nd DPP4 and seprase measurements ($p = 0.720$ and $p = 0.772$, respectively).

3.4. Association of plasma seprase and DPP4 levels with survival

Follow-up data were available from 346 cancer patients. Survival time was measured from the date that the blood specimen was collected to time of death or last follow-up visit. Median follow-up duration was 645 (IQR = 1622) days; 115 (33.2%) of the cases were alive at the last follow-up visit. In Cox regression analysis of all cancer cases combined, lower DPP4 was associated with significantly shorter survival (hazard ratio [HR] 0.695, 95% confidence interval [CI] 0.545–0.886 for the log-transformed variable, $p = 0.003$). Likewise, when patients with all types of cancer were combined and grouped based on their DPP4 level being above or below the median, patients with lower DPP4 levels had shorter survival compared to those with higher DPP4 levels ($p = 0.021$ for Log Rank test comparing groups above or below median DPP4 level of 3.43 $\mu\text{g/mL}$; Fig. 4A). In contrast, there was no significant association between survival and plasma seprase levels (HR 0.921, 95% CI 0.740–1.147, $p = 0.463$). However, when metastatic cases were excluded from the analysis, lower seprase was associated with decreased survival (HR 0.637, 95% CI 0.423–0.960 for the log-transformed variable, $p = 0.031$; $p = 0.037$ for the Log Rank test comparing groups above or below median seprase level of 0.35 $\mu\text{g/mL}$, $n = 151$).

Fig. 2. Boxplots of plasma DPP4 levels (panel A) and seprase levels (panel B) in cancers with different stage. Numbers represent median (interquartile range). In panel A, “*” denotes $p < 0.001$ for comparison with DPP4 level in Stage III cancer. In panel B, “*” denotes $p = 0.017$ and “**” denotes $p = 0.002$ for comparison with seprase level in Stage III cancer.

Fig. 3. Scatter-plots of repeat DPP4 (Panel A) and seprase (Panel B) measurements in cancer patients before and after surgery.

3.5. Cancer subgroup analysis of plasma seprase and DPP4

Among hematological malignancies, DPP4/seprase levels were similar in leukemia ($n = 9$), lymphoma ($n = 15$) and multiple myeloma ($n = 4$) cases and they were analyzed together. Older age was associated with significantly higher seprase levels ($p = 0.001$), but not DPP4 ($p = 0.786$).

Head and neck malignancies consisted of 20 cases of oropharyngeal malignancies, 12 laryngeal cancers and 8 thyroid cancer cases. DPP4/seprase levels were similar among different cancer types. Patients with higher seprase levels had better survival (HR 0.486, 95% CI 0.213–1.110 for log-transformed variable, $p = 0.087$; $p = 0.039$ for Log Rank test comparing groups above or below median seprase level of $0.41 \mu\text{g/mL}$; $n = 38$; Fig. 4B). Similarly, head and neck cancer patients with higher DPP4 levels had better survival (HR 0.252, 95% CI 0.079–0.806 for the log-transformed variable, $p = 0.020$; $n = 38$) (Fig. 4C).

Lung malignancies consisted of 67 non-small cell and 16 small cell carcinomas. There were no significant

differences in DPP4 and seprase levels between these tumor types. Older age was associated with lower DPP4 levels ($p = 0.025$). DPP4 level in stage III cancers ($2.30 \pm 0.79 \mu\text{g/mL}$; $n = 12$) was significantly lower compared with stage I ($4.77 \pm 0.38 \mu\text{g/mL}$, $p = 0.031$; $n = 4$) and stage IV ($3.95 \pm 1.67 \mu\text{g/mL}$, $p = 0.009$; $n = 51$) cancers. There were no significant associations between lower DPP4 and seprase levels, and shorter survival ($n = 64$).

In gynecologic cancers, smaller tumors (T1) had higher DPP4 levels compared with T2–4 tumors ($p < 0.001$). Similarly, stage I tumors had significantly higher DPP4 levels than other stages ($p < 0.001$). There was no significant association between DPP4 and seprase levels and survival ($n = 79$).

Older age was associated with lower seprase levels in patients with colorectal cancer ($p = 0.016$). In contrast to other cancers, colorectal cancer patients with higher plasma seprase levels (based on various cut-off points ranging from 60th to 80th percentile) had worse survival when compared using Log Rank test ($p = 0.003$, highest significance using a cut-off seprase level of $0.64 \mu\text{g/mL}$ [70th percentile]; $n = 47$; Fig. 4D).

Fig. 4. Survival in cancer patients grouped based on median DPP4 and seprase levels (i.e. lower 50% compared with upper 50%). All *p* values are from Log Rank test comparing survival between the two groups. A) All cancer patients combined ($n = 346$), with patients with DPP4 levels below $3.43 \mu\text{g/mL}$ (solid line) versus those with DPP4 levels above this median cut-off point (dotted line). B) Head and Neck cancer ($n = 38$), with patients with seprase levels below $0.41 \mu\text{g/mL}$ (solid line) compared with those with seprase levels above this level (dotted line). C) Head and Neck cancer ($n = 38$), with patients with DPP4 levels below $3.84 \mu\text{g/mL}$ (solid line) versus those with DPP4 levels above this median cut-off point (dotted line). D) Colorectal cancer ($n = 47$), with patients with seprase levels below $0.64 \mu\text{g/mL}$ (solid line) compared with those with higher seprase levels (dotted line). Censored cases (patients who had a shorter follow-up duration and were still alive at the last follow-up) are denoted by plus sign (+).

Other gastroenterological malignancies included 19 gastro-esophageal tumors and 8 liver/pancreas cancer cases. Liver/pancreas cancers had significantly higher DPP4 ($5.99 \pm 3.05 \mu\text{g/mL}$) and seprase ($0.68 \pm 0.16 \mu\text{g/mL}$) levels compared with gastro-esophageal cancers (DPP4: $3.19 \pm 1.64 \mu\text{g/mL}$, $p = 0.009$; seprase: $0.39 \pm 0.20 \mu\text{g/mL}$, $p = 0.008$). Also, seprase levels in gastro-esophageal cancers were significantly lower compared with colorectal tumors ($p = 0.020$).

Overall in the subgroup analyses, no statistically significant association was noted between DPP4/seprase levels and age, tumor size, lymph node involvement, metastasis and staging in breast, head and neck and prostate malignancies. Likewise, there was no association between DPP4/seprase levels and gender in head and neck, lung and colorectal malignancies. No effect between DPP4/seprase levels and survival was noted in breast ($n = 34$) and hematological malignancies ($n = 15$). Finally, in gynecologic cancers, there was no association between DPP4/seprase levels and patients' age, CA125 levels and circulating tumor cell counts at time of diagnosis [11].

4. Discussion

There has been considerable recent interest in the role of DPP4 in various pathophysiologic processes, including development of novel treatments for diabetes, cancer and inflammatory disease [3,22,44,46]. Another member of the DPP family, seprase, is emerging as an interesting target because of its high concentrations in solid tumors and its suggested function in tumor invasion [23,34]. Seprase has also been suggested to be a promising diagnostic and therapeutic target in cancer [1,4,6,28,33,36,40,45]. Although seprase has recently been isolated from plasma, the origin and clinical relevance of circulating seprase in cancer has not been addressed [30,33].

In this study, an ELISA for measurement of seprase and DPP4 in plasma was developed and validated to be sensitive and specific, and yield reproducible results. Our study measured plasma seprase as an antigen using monoclonal antibodies known to bind untruncated monomers and dimers of seprase. Seprase was

first isolated from blood as an enzymatically-active homodimer consisting of two ~97-KDa monomers [30]. Given this, and our preliminary observation that our ELISA measurements correlated with enzymatic measurements in a limited experiment, we believe what our ELISA measured in this study mostly consisted of enzymatically-active untruncated seprase dimers in plasma.

Seprase and DPP4 levels measured in subsequent blood samples of a subset of patients obtained after surgical tumor removal/debulking highly correlated with levels in initial preoperative samples; this observation can be interpreted to suggest that either the tumor contribution to circulating seprase and DPP4 is small or the half-life of circulating enzymes is long. The current study demonstrates new findings with regards to plasma seprase concentrations in cancer and confirms previously reported correlations regarding DPP4. DPP4 levels in plasma of healthy adults have been previously reported to be approximately 7 $\mu\text{g/mL}$ (range 0.1–15.6 $\mu\text{g/mL}$) [8,20]. The measured mean DPP4 level in our plasma samples from healthy adults ($5.69 \pm 1.47 \mu\text{g/mL}$) was in line with previous reports. Some variations among this and previously reported levels of DPP4 is expected, given variable and non-standardized methodologies. The mean plasma seprase level was considerably lower ($0.66 \pm 0.19 \mu\text{g/mL}$ in healthy subjects) compared with DPP4 level in the same individuals ($p < 0.001$). Previous reports indicate that approximately 90% of the DPP enzymatic activity in human plasma can be attributed to plasma DPP4; the source of the remaining 10% of activity has been a mystery [8]. Based on the current study, seprase appears to account for the bulk of the remaining DPP enzymatic activity in plasma. Additionally, variations of plasma DPP4 with age and gender have been reported [20]. In the current study, plasma DPP4 and seprase levels were found to be higher in healthy male than female subjects, as well as in younger compared with older individuals (with all cases combined), both in line with previous DPP4 reports [20].

In the current study of 561 cancer patients and 139 healthy subjects, we observed lower plasma DPP4 levels in most patients with cancer. We also demonstrated that plasma seprase levels in cancer patients were significantly lower than in healthy subjects, with gynecologic, hematologic, lung and head and neck cancer patients displaying particularly low plasma seprase levels. These findings cannot be explained by differences in patient age or gender, although lack of comorbidity data precluded us from exploring other pos-

sible reasons for this observation. This was an unexpected observation in light of extensive evidence showing high expression and concentration of seprase in tumor tissue specimens [7,10,12,13,21,25,28,32,35,47]. Consistent with the finding of this study (as initially presented in 2010 as part of the first author's doctoral dissertation [26]), a recent report showed that serum seprase levels were lower in colorectal cancer patients compared with healthy volunteers [43]. Wild et al. used ELISA to measure seprase along with other tumor markers as a potential test for early detection of cancer, and they reported that serum levels of carcinoembryonic antigen (CEA) and seprase were superior to 20 other serum biomarkers for this purpose [43]. However, in their study, serum seprase levels were expressed in arbitrary units as opposed to our study, which measured the absolute level of plasma seprase.

The physiologic sources of seprase and DPP4 remain to be determined. Evidence suggests the hepatobiliary system and immune cells as the primary physiologic sources of plasma DPP4. Some studies reported increased plasma DPP4 levels in patients with tumors arising from these tissues [8]. Interestingly, relatively high plasma seprase (as well as DPP4) levels were observed in patients with liver/pancreatic cancers in the present study, which lends support to these organs being among physiologic sources of plasma seprase.

Regardless of the source, certain disease markers are known to decrease during disease progression. Hence, the lower level of plasma seprase seen in cancer patients could possibly be a phenomenon comparable to decreased plasma level of negative acute-phase proteins seen in inflammatory processes and malignancy (e.g., low plasma levels of transferrin, albumin, and inter-alpha-trypsin inhibitors) [9,38], or related to other speculated processes, such as active uptake of seprase from plasma by tumor cells or the reticuloendothelial system (alone, or in combination with other molecules such as auto-antibodies).

In the current study, cancer patients with higher plasma levels of DPP4 experienced significantly longer survival; a similar trend was observed with plasma seprase, which became statistically significant once metastatic cases were excluded. Altogether, our data suggest that lower plasma DPP4 and seprase levels are associated with decreased survival, particularly in stage I, II and III cancer, and progression to metastatic cancer is often associated with a late surge in plasma seprase levels, as can be seen in Fig. 2. Given the observation that seprase is present under physiologic conditions in plasma of healthy subjects, and the previously-reported ob-

Fig. 5. “Dual source” model of plasma seprase in cancer, from yet-to-be-identified physiologic source(s) (the left panel, depicted by question marks) and seprase-expressing tumor cells (the right panel). It can be speculated that as cancer progresses into metastatic disease, the plasma seprase derived from the physiologic source(s) declines while the seprase derived from tumor increases.

servation that membrane-bound seprase is cleaved and released from tumor cells into culture medium [4], it is likely that plasma seprase is derived from two distinct sources in cancer patients: yet-to-be-identified physiologic source(s), and the tumor; which we propose here as the “dual source” model of seprase in blood circulation (Fig. 5). Hence we speculate that as disease progresses, the plasma level of seprase from physiologic source(s) decreases similar to negative acute-phase proteins. Then, following metastasis, while the physiologically-derived seprase in plasma continues to decline, tumor-derived seprase increases (i.e. more readily enters circulation with tumor cells exposed to blood circulation) and emerges as the dominant source of plasma seprase. The net result of these changes can lead to an initial decrease in plasma seprase level followed by a late increase in metastatic disease, as seen in our study and the report by Wild et al. [43]. Seprase can thus be viewed as a useful disease marker in both early detection of cancers (plasma levels decrease), and as a marker of metastasis (plasma levels increase).

In conclusion, this study concurrently evaluated plasma levels of seprase and DPP4 in healthy subjects and a large number of cancer patients. Contrary to expectations and similar to previously reported data on DPP4 and a recent study on seprase, plasma levels of seprase were lower in cancer patients compared with healthy subjects; lower plasma levels were associated with shorter survival, suggesting that circulating seprase might represent an inverse tumor marker. The physiologic source of seprase in blood and the reason for lower levels in cancer patients remains to be identified. This study will serve as the basis for further anal-

yses of the prognostic significance of plasma seprase in cancer and its role as a disease marker.

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Disclosure of potential conflicts of interest

WTC is the inventor of patent applications on anti-DPP4 and anti-seprase antibodies, and the president of Vitatex Inc.

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