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

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Abstract. 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 prollyl peptidase (DPP) family, DPP4, are membrane-bound serine proteases \cite{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 \cite{16,17}. Their coupled expression in neoplastic cells has been reported \cite{2}. Seprase is an integral membrane protein and it lacks a secretory signal peptide. Seprase is shed from cells as soluble forms \textit{in vitro}; further activation of its proteolytic activity through metalloprotease-mediated truncation has been described \cite{4}. Soluble seprase has also been identified in blood, with the initial description of its role in coagulation as antiplasmin-cleaving enzyme (APCE) \cite{30}. Other suggested natural substrates of seprase include neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY \cite{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 \cite{5,7,13,21,23,25,28,29,35,39,47}. Seprase and DPP4 levels were measured in plasma samples from 139 healthy volunteers and 561 cancer patients. Mean seprase and DPP4 levels were 0.51 ± 0.30 and 4.65 ± 6.37 μg/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 μg/mL, \textit{p} < 0.001 for DPP4; 0.46 versus 0.66 μg/mL, \textit{p} < 0.001 for seprase). Higher DPP4 was associated with better survival in all cancers combined (\textit{n} = 346) as well as in head and neck malignancies (\textit{n} = 38). Higher seprase was associated with better survival in all non-metastatic cancers combined (\textit{n} = 151) as well as head and neck malignancies, but worse survival in colorectal cancers (\textit{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 prollyl peptidase (DPP) family, DPP4, are membrane-bound serine proteases \cite{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 \cite{16,17}. Their coupled expression in neoplastic cells has been reported \cite{2}. Seprase is an integral membrane protein and it lacks a secretory signal peptide. Seprase is shed from cells as soluble forms \textit{in vitro}; further activation of its proteolytic activity through metalloprotease-mediated truncation has been described \cite{4}. Soluble seprase has also been identified in blood, with the initial description of its role in coagulation as antiplasmin-cleaving enzyme (APCE) \cite{30}. Other suggested natural substrates of seprase include neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY \cite{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 \cite{5,7,13,21,23,25,28,29,35,39,47}.

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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 FAPα/seprase. Although preclinical studies on F19 immunonjugates 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 Igκ 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 using 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 antibo-
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 readings 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 fusion 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 μg/mL for seprase and 0.6-19.2 μg/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 μg r-seprase to plasma samples containing 0.45 μg seprase as previously measured by ELISA, the total measured seprase increased to 2.20 μg (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 μg rDPP4 to plasma samples containing 6.36 μg DPP4, the total measured DPP4 increased to 9.05 μg (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
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 ± 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
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 ± 6.37 μg/mL, respectively. Two gynecologic cancer patients had extremely high DPP4 levels in excess of 100 μg/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 ± 2.24 μg/mL; mean seprase level was unchanged). The DPP4 and seprase levels were correlated with each other, but several outliers were present as well (R² = 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 ± 0.18 μg/mL, respectively, p = 0.015). Similarly, DPP4 levels were higher in healthy male versus female subjects, (6.31 ± 1.21 μg/mL versus 5.65 ± 1.48 μg/mL, respectively, p = 0.039).

Plasma seprase and DPP4 levels were significantly lower in cancer patients compared with healthy subjects (3.95 ± 2.29 μg/mL versus 5.69 ± 1.47 μg/mL, respectively, p < 0.001 for DPP4; 0.47 ± 0.30 versus 0.66 ± 0.19 μg/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 ± 1.65 μg/mL versus 5.74 ± 1.44 μg/mL, respectively, p < 0.001 for DPP4; 0.45 ± 0.25 versus 0.67 ± 0.19 μg/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).
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 µg/mL, respectively (p = 0.101). Seprase levels in cancer patients with and without metastasis were 0.50 ± 0.41 and 0.43 ± 0.22 µg/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 (R² = 0.684, p < 0.001 for DPP4 [Fig. 3A], and R² = 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 µg/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 µg/mL, n = 151).
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 \(\mu g/mL; n = 38\); 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 \((2.30 ± 0.79 \mu g/mL; n = 12)\) was significantly lower compared with stage I \((4.77 ± 0.38 \mu g/mL, p = 0.031; n = 4)\) and stage IV \((3.95 ± 1.67 \mu g/mL, p = 0.009; n = 51)\) 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 g/mL [70th percentile]; n = 47; Fig. 4D).
AB

CD

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 µg/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 µg/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 µg/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 µg/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 ± 3.05 µg/mL) and seprase (0.68 ± 0.16 µg/mL) levels compared with gastro-esophageal cancers (DPP4: 3.19 ± 1.64 µg/mL, p = 0.009; seprase: 0.39 ± 0.20 µg/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 μg/mL (range 0.1–15.6 μg/mL) [8,20]. The measured mean DPP4 level in our plasma samples from healthy adults (5.69 ± 1.47 μg/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 ± 0.19 μg/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 possible 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.

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 analyses 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.

References

[1] A. Aoyama and W.-T. Chen, A 170-kDa membrane-bound protease is associated with the expression of invasiveness by human malignant melanoma cells, Proc Natl Acad Sci USA 87 (1990), 8296–8300.
[2] E. Balazsiova, P. Busek, J. Stremenova, L. Sromova, E. Krepela, L. Lizcova and A. Sedo, Coupled expression of dipeptidyl peptidase-IV and fibroblast activation protein-alpha in transformed astrocytic cells, Mol Cell Biochem 354 (2011), 283–289.

[3] P. Busek, J. Stremenova and A. Sedo, Dipeptidyl peptidase-IV enzymatic activity bearing molecules in human brain tumors—good or evil? Front Biosci 13 (2008), 2319–2326.

[4] D. Chen, A. Kennedy, J.Y. Wang, W. Zeng, Q. Zhao, M. Pearl, M. Zhang, Z. Suo, J.M. Nesland, Y. Qiao, A.K. Ng, N. Hirashima, T. Yamane, Y. Mori, M. Mitsumata, G. Ghersi and W.T. Chen, Activation of EDTA-resistant gelatinases in malignant human tumors, Cancer Res 66 (2006), 9977–9985.

[5] J.D. Cheng, R.L. Dunbrack, Jr., M. Valianou, A. Rogatko, R.K. Alpaugh and L.M. Weiner, Promotion of tumor growth by murine fibroblast activation protein, a serine protease, in an animal model, Cancer Res 62 (2002), 4767–4772.

[6] V.I. Christiansen, K.W. Jackson, K.N. Lee and P.A. McKee, Effect of fibroblast activation protein and alpha2-antiplasmin cleaving enzyme on collagen types I, III, and IV, Arch Biochem Biophys 457 (2007), 177–186.

[7] S.J. Cohen, R.K. Alpaugh, I. Palazzo, N.J. Meropol, A. Rogatko, Z. Xu, J.P. Hoffman, L.M. Weiner and J.D. Cheng, Fibroblast activation protein and its relationship to clinical outcome in pancreatic adenocarcinoma, Pancreas 37 (2008), 154–158.

[8] O.J. Cordero, F.J. Salgado and M. Nogueira, On the origin of serum CD26 and its altered concentration in cancer patients, Cancer Immunol Immunother 58 (2009), 1723–1747.

[9] M. Devi, P. Rouet, M. Scotta, L. Faye, M. Hiron, J.P. Lebret and J.P. Sallier, Human inter-alpha-inhibitor family in inflammation: simultaneous synthesis of positive and negative acute-phase proteins, Biochem J 292 (Pt 2) (1993), 485–492.

[10] H. Dolznig, N. Schweifer, C. Puri, H.O. Lee, W.T. Chen, M. Javidroozi, S. Iwasa, K. Okada, W.T. Chen, X. Jin, T. Yamane, A. Ooi and M. Mitsumata, Increased expression of seprase, a membrane-type serine protease, is associated with lymph node metastasis in human colorectal cancer, Cancer Lett 227 (2005), 229–236.

[11] T. Fan, Q. Zhao, J.J. Chen, W.T. Chen and M.L. Pearl, Clinical significance of circulating tumor cells detected by an invasion assay in peripheral blood of patients with ovarian cancer, Clin Exp Metastasis 28 (2011), 567–579.

[12] S. Iwasa, K. Okada, W.T. Chen, X. Jin, T. Yamane, A. Ooi and M. Mitsumata, Increased expression of seprase, a membrane-type serine protease, is associated with lymph node metastasis in human colorectal cancer, Cancer Lett 227 (2005), 229–236.

[13] M. Javidroozi, A.H. Farzaneh, M. Javidroozi, A.H. Farzaneh, A. Gopinathan, D.A. Tuveson and D.T. Fearon, Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha, Science 330 (2010), 827–830.

[14] A. Kennedy, H. Dong, E. Lee and J.D. Cheng, Elevated expression of seprase and promotion of an invasive phenotype by collagenous matrices in ovarian cancer cell lines, Clin Cancer Res 12 (2006), 2329–2337.

[15] M. Kraman, P.J. Bambrough, J.N. Arnold, E. Roberts, L. Magiera, J.O. Jones, A. Gopinathan, D.A. Tuveson and D.T. Fearon, Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha, Science 330 (2010), 827–830.

[16] S. Iwasa, K. Okada, W.T. Chen, X. Jin, T. Yamane, A. Ooi and M. Mitsumata, Increased expression of seprase, a membrane-type serine protease, is associated with lymph node metastasis in human colorectal cancer, Cancer Lett 227 (2005), 229–236.

[17] M. Javidroozi, A.H. Farzaneh, M. Javidroozi, A.H. Farzaneh, A. Gopinathan, D.A. Tuveson and D.T. Fearon, Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha, Science 330 (2010), 827–830.
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[33] K. Narra, S.R. Mullins, H.O. Lee, B. Strzemkowski-Brun, K. Magalong, V.I. Christiansen, P.A. McKee, B. Egleston, S.J. Cohen, L.M. Weiner, N.J. Meropol and J.D. Cheng, Phase II trial of single agent Val-boroPro (Talabostat) inhibiting Fibroblast Activation Protein in patients with metastatic colorectal cancer, *Cancer Biol Ther* **6** (2007), 1691–1699.

[34] P. O’Brien and B.F. O’Connor, Seprase: an overview of an important matrix serine protease, *Biochim Biophys Acta* **1784** (2008), 1130–1145.

[35] K. Okada, W.-T. Chen, S. Iwasa, X. Jin, T. Yamane, A. Ooi, and M. Mitsumata, Seprase, a membrane-type serine protease, has different expression patterns in intestinal- and diffuse-type gastric cancer, *Oncology* **65** (2003), 363–370.

[36] E. Ostermann, P. Garin-Chesa, K.H. Heider, M. Kalat, H. Lamche, C. Puri, D. Kerjaschki, W.J. Rettig and G.R. Adolf, Effective immunoconjugate therapy in cancer models targeting a serine protease of tumor fibroblasts, *Clin Cancer Res* **14** (2008), 4584–4592.

[37] M.L. Pineiro-Sanchez, L.A. Goldstein, J. Dodd, L. Howard, Y. Yeh, H. Tran, W.S. Argraves and W.T. Chen, Identification of the 170-kDa melanoma membrane-bound gelatinase (seprase) as a serine integral membrane protease, *J Biol Chem* **272** (1997), 7595–7601.

[38] R.F. Ritchie, G.E. Palomaki, L.M. Neveux, O. Navolotskaia, T.B. Ledue and W.Y. Craig, Reference distributions for the negative acute-phase serum proteins, albumin, transferrin and transthyretin: a practical, simple and clinically relevant approach in a large cohort, *J Clin Lab Anal* **13** (1999), 273–279.

[39] S. Saigusa, Y. Tsuboyama, K. Tanaka, T. Yokoe, Y. Okugawa, H. Fujikawa, K. Matsusita, M. Kawamura, Y. Inoue, C. Miki and M. Kusunoki, Cancer-associated fibroblasts correlate with poor prognosis in rectal cancer after chemoradiotherapy, *Int J Oncol* **38** (2011), 655–663.

[40] A.M. Scott, G. Wiseman, S. Welt, A. Adjei, F.T. Lee, W. Hopkins, C.R. Divgi, L.H. Hansson, P. Mitchell, D.N. Gansen, S.M. Larson, J.N. Ingle, E.W. Hoffman, P. Tanswell, G. Ritter, L.S. Cohen, P. Bette, L. Arvay, A. Amelsberg, D. Vlock, W.J. Rettig and L.J. Old, A Phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer, *Clin Cancer Res* **9** (2003), 1639–1647.

[41] X.M. Wang, D.M. Yu, G.W. McCaughaun and M.D. Gorrell, Fibroblast activation protein increases apoptosis, cell adhesion, and migration by the LX-2 human stellate cell line, *Hepatology* **42** (2005), 935–945.

[42] S. Welt, C.R. Divgi, A.M. Scott, P. Garin-Chesa, R.D. Finn, M. Graham, E.A. Carswell, A. Cohen, S.M. Larson, L.J. Old et al., Antibody targeting in metastatic colon cancer: a phase I study of monoclonal antibody F19 against a cell-surface protein of reactive tumor stromal fibroblasts, *J Clin Oncol* **12** (1994), 1193–1203.

[43] N. Wild, H. Andres, W. Rollinger, F. Krause, P. Dilba, M. Tacke and J. Karl, A combination of serum markers for the early detection of colorectal cancer, *Clin Cancer Res* **16** (2010), 6111–6121.

[44] R. Yazbeck, G.S. Howarth and C.A. Abbott, Dipeptidyl peptidase inhibitors, an emerging drug class for inflammatory disease? *Trends Pharmacol Sci* **30** (2009), 600–607.

[45] Y.M. Yi, G. Zhang, J. Zeng, S.C. Huang, L.L. Li, R. Fang, G.M. Jiang, X.Z. Bu, S.H. Cai and J. Du, A new tumor vaccine: FAPtau-MT elicits effective antitumor response by targeting indolamine2,3-dioxygenase in antigen presenting cells, *Cancer Biol Ther* **11** (2011), 866–873.

[46] D.M. Yu, T.W. Yao, S. Chowdhury, N.A. Nadvi, B. Osborne, W.B. Church, G.W. McCaughaun and M.D. Gorrell, The dipeptidyl peptidase IV family in cancer and cell biology, *FEBS J* **277** (2011), 1126–1144.

[47] M.Z. Zhang, Y.H. Qiao, J.M. Nesland, C. Trope, A. Kennedy, W.T. Chen and Z.H. Suo, Expression of seprase in effusions from patients with epithelial ovarian carcinoma, *Chin Med J (Engl)* **120** (2007), 663–668.