Stony Brook University

Association of Blood DPP5 Level with Cancer Type, Stage, and Survival

A Dissertation Presented

by

Mazyar Javid Roozi

to

The Graduate School

In Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Molecular and Cellular Biology

(Immunology and Pathology)

Stony Brook University

August 2010

Stony Brook University

The Graduate School

Mazyar Javid Roozi

We, the dissertation committee for the above candidate for the Doctor of Philosophy degree, hereby recommend acceptance of this dissertation.

Wen-Tien Chen, Ph.D. – Dissertation Advisor
Professor, Department of Medicine

Howard B. Fleit, Ph.D. – Chairperson of Defense
Associate Professor, Department of Pathology

Sanford R. Simon, Ph.D.
Professor, Departments of Biochemistry, Cell Biology, and Pathology

Stanley H. Zucker, M.D.
Professor, Department of Medicine, Stony Brook University

This dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

Association of Blood DPP5 Level with Cancer Type, Stage, and Survival

by

Mazyar Javid Roozi

Doctor of Philosophy

in

Molecular and Cellular Biology

(Immunology and Pathology)

Stony Brook University

2010

Introduction: Membrane-bound dipeptidyl peptidase 5 (DPP5) has been linked to tumor invasion and metastasis processes. Its recent isolation as a soluble protein from plasma may indicate a potential role as a biomarker and prognostic factor for tumors, and as a potential therapeutic target. This study aimed to measure the plasma levels of DPP5 and the related member of the family, DPP4, in healthy subjects and cancer patients, and to determine their clinical significance.

Methods: Enzyme-linked immunosorbent assays (ELISAs) for measuring plasma DPP5 and DPP4 were developed and clinical correlations were made.

Results: ELISAs were validated to be sensitive and specific. In the 747 plasma samples studied, mean DPP5 and DPP4 levels were 0.51±0.30 and 4.65±6.37 μg/mL, respectively. Plasma DPP5 levels were highly correlated with DPP4 levels. Plasma DPP5 and DPP4 levels were significantly lower in cancer

patients compared with healthy subjects (4.38 versus 5.65 μg/mL μg/mL, $p<0.001$ for DPP4; 0.46 versus 0.66 μg/mL, $p<0.001$ for DPP5). DPP5 and DPP4 levels were higher in younger patients and those with earlier-stage cancers, but also in metastatic cancers; stage III cancer patients had the lowest plasma levels. Cancer patients with low DPP4 levels had shorter survival than those with higher DPP4 levels ($p=0.001$). There was also a trend toward shorter survival in cancer patients with low DPP5 levels in various cancer types, with the exception of colorectal cancers. An *in vitro* model indicated that approximately 80% of DPP5 released from the cells into conditioned medium is free (not associated with membranes), and serine protease inhibitors interfere with release of DPP5 into medium.

Conclusions: This study demonstrates that plasma DPP5 and DPP4 levels are reproducible parameters to correlate with disease status, with lower levels noted in cancer patients compared with healthy subjects; but higher levels in metastatic cancers compared with non-metastatic cancers. These findings support a prognostic role for plasma DPP5 and DPP4 levels and they should be considered in attempts to use DPP5 as a therapeutic target to hinder cancer progression. An explanation of low levels of plasma DPP5 in cancer despite previously-reported high level of local expression remains to be determined.

To my wife, whose love and support was pivotal for completing this work,
and to my daughters, who have been my joy and inspiration

Table of Contents

List of Tables

List of Figures

List of Abbreviations

- a.a.: amino acid
- AEBSF: 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
- ANOVA: analysis of variance
- APCE: antiplasmin cleaving enzyme (also: DPP5, FAP-α, seprase)
- BSA: bovine serum albumin
- CCC medium: cancer cell culture medium
- CD26: cluster of differentiation 26 (also: DPP4)
- CI: confidence interval
- CV: coefficient of variation
- dH_2O: distilled water
- DMEM: Dulbecco's modified Eagle's medium
- DMSO: Dimethyl sulfoxide
- DNA: deoxyribonucleic acid
- DPP4: dipeptidyl peptidase 4 (also: CD26)
- DPP5: dipeptidyl peptidase 5 (also: APCE, FAP-α, seprase)
- DSS: DPP4 Standard Stock
- EACA: epsilon-aminocaproic acid
- ECM: extracellular matrix
- EDTA: ethylenediaminetetraacetic acid
- ELISA: enzyme-linked immunosorbent assay
- ER: endoplasmic reticulum
- GWSYG: serine protease motif (Gly-Trp-Ser-Tyr-Gly)
- kb: kilobase
- kDa: Kilo Dalton
- FAP-α: fibroblast activating protein (also: APCE, DPP5, seprase)
- FBS: fetal bovine serum
- GI: gastrointestinal
- HR: hazard ratio

- IHC: immunohistochemistry
- IRB: institutional review board
- mAb: monoclonal antibody
- mRNA: messenger RNA
- MMP: matrix metalloproteinase
- NEM: N-Ethylmaleimide
- OG: n-octyl p-D-glucopyranoside
- PBS: phosphate buffered saline
- PBST: PBS with 0.05% Tween-20
- rDPP4: recombinant soluble (free) DPP4
- rDPP5: recombinant soluble (free) DPP5
- RNA: ribonucleic acid
- ROC: receiver-operative characteristics
- RT-PCR: Real-time polymerase chain reaction
- SCC: squamous cell carcinoma
- SD: standard deviation
- SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
- SSS: DPP5 Standard Stock
- TGF-β_1: Transforming growth factor beta-1
- TNM: tumor, node, metastasis (as used in cancer staging)
- TT: Trizma-Tween-20

Acknowledgments

I would like to express my utmost gratitude to my mentor and advisor, Dr. Wen-Tien Chen, whose continuous support and insightful advice throughout my work were essential in completing my study and my project. He took every opportunity to go above and beyond what is normally expected from an advisor, and I am forever indebted to his exemplary mentorship.

I am grateful to my thesis and defense committee members, Dr Howard Fleit (who served as the chairman of the committee), Dr Sanford Simon, and Dr Stanley H. Zucker, for all their advice, suggestions, and support throughout my work. Dr Zucker also played a tremendous role by providing extensive clinical data and contributing to my thesis paper.

I would like to thank Dr. Donghai Chen for his tremendous contributions to this study, including but not limited to developing r-DPP4 and r-DPP5 vectors, producing monoclonal antibodies and protocols for producing and purification of recombinant proteins. I am grateful to Dr. Huan Dong for his assistance in production and purification of recombinant proteins. Dr. Tina Fan kindly provided some additional clinical and laboratory data. I thank Qiang Zhao for her invaluable assistance in various stages of laboratory assays. Last but not least, my classmates, colleagues and friends, Shaun Tulley and Azadeh Jadali provided several extremely helpful insights and suggestions as well as continuous support and encouragement during conduction of this study.

This dissertation was supported in part by grants from the National Institutes of Health; grants M01 RR010710, R01CA0039077 and R01EB002065 to Dr. Wen-Tien Chen.

The text of this dissertation in part is a reprint of a manuscript co-authored by Dr. Wen-Tien Chen and Dr. Stanley Zucker as it has been submitted for publication in a peer-reviewed journal.

BACKGROUND

Human dipeptidyl prolyl peptidase 5 (DPP5; Seprase; fibroblast activation protein [FAP]; Gene ID: 2191, accession number: NP_004451; protein accession number: Q12884, gi:160113236) was first identified as a 170 kDa homodimeric membrane-bound glycoprotein consisting of two 97 kDa subunits. DPP5 and the other well-known member of the dipeptidyl prolyl peptidase (DPP) family, DPP4 (CD26), are type II transmembrane serine proteases. DPP5 possesses dipeptidyl dipeptidase and gelatinase activities. (Henry et al., 2007; Busek et al., 2008; Havre et al., 2008; O'Brien and O'Connor, 2008) (Figure 1). Both proteases share nearly identical domain structures and catalytic sites; DPP5 and DPP4 share 50% overall amino acid sequence homology and 70% amino acid sequence homology in the catalytic domain (Goldstein et al., 1997). 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 DPP5 are known to form a hetero-oligomer in a proteolytic complex on cell membranes, which plays a role in the invasion of cells in collagenous matrices (Ghersi et al., 2002; Ghersi et al., 2006; Chen et al., 2003; Ghersi et al., 2003).

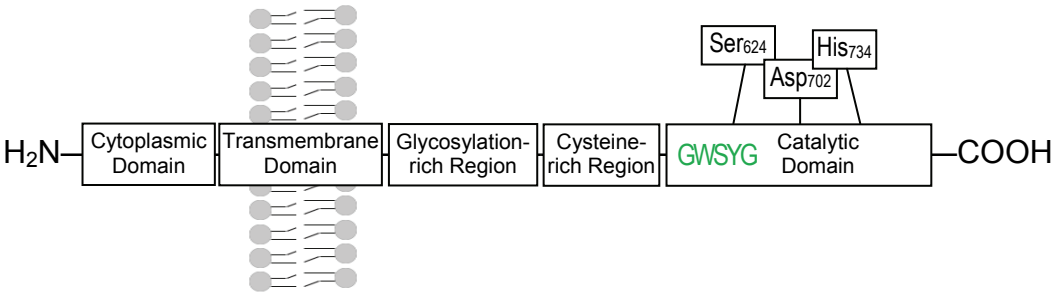

Figure 1- Domain structure of DPP5. A single 97-kDa subunit is depicted. GWSYG is the serine protease motif. The a.a. residues Ser_{624}, Asp_{702}, and His_{734} form the catalytic site of the protease, which is active in homodimeric form of DPP5. Sizes of the boxes are not proportional to the actual length of the domains (Modified from: (Chen and Kelly, 2003)).

Physiologic function(s) and substrate(s) of DPP5 have remained mostly elusive. Its gelatinase activity suggests a role in tissue remodeling and invasion. DPP5 is able to cleave gelatin and human collagen type I (but unable to cleave human fibronectin, laminin, or collagen type IV) in zymography experiments (Pineiro-Sanchez et al., 1997). Another study has shown that DPP5 works together with other proteases (namely MMP-1) to cleave partially degraded or denatured collagen I and III as ECM is invaded (Christiansen et al., 2007). The DPP activity has been more widely studied, since it is the defining feature of the members of this family including DPP4, DPP2, DPP7, DPP8 and DPP9 (Kelly, 2005). These enzymes are all capable of cleaving N-terminus peptides with proline as the penultimate amino acid (Park et al., 1999).

DPP5 expression has been detected in the human malignant melanoma LOX tumor cells (especially localized on the invadopodia) (Monsky et al., 1994; Aoyama and Chen, 1990) as well as numerous other malignant tumors (mainly carcinomas, localized to the tumor cells as well as the reactive stroma/fibroblasts) and during embryogenesis and wound healing (Scanlan et al., 1994; Park et al., 1999). Tumors with high DPP5 expression include lung, breast, colorectal, gastric, ovarian, head and neck, bladder, skin and pancreatic tumors as well as some soft-tissue tumors and sarcomas (Dolznig et al., 2005; Kennedy et al., 2009; Dohi et al., 2009; Zhi et al., 2010). It has been suggested that while some epithelial tumor cells express low levels of DPP5 *per se*, they can be induced to express much higher levels of DPP5 (Kennedy et al., 2009). DPP5 expression has been identified in other contexts including chronic liver disease and arthritis (Wang et al., 2008; Bauer et al., 2006; Milner et al., 2006) as well as bone marrow mesenchymal stromal cells (Monsky et al., 1994; Bae et al., 2008).

An increasing body of evidence suggests that membrane-bound DPP5 expression in various solid tumors is associated with an invasive phenotype and poor prognosis (Henry et al., 2007; Cohen et al., 2008; Kennedy et al., 2009; Okada et al., 2003; Iwasa et al., 2005; Garin-Chesa et al., 1990; Zhang et al., 2007; Chen, 2003; Kelly, 2005; Monsky et al., 1994; Wolf et al., 2008; O'Brien and O'Connor, 2008), making DPP5 an attractive subject for study of tumor cell

invasion, as an antigen for tumor imaging, and as a potential therapeutic target (Pure, 2009). In gastric cancer, stromal expression of DPP5 was more abundant in intestinal type versus the diffuse type and it correlated with liver or lymph node metastasis (Okada et al., 2003). In an animal model of breast cancer, it was noted that engineered expression of DPP5 resulted in fast growing and highly vascular tumors (Huang et al., 2004). Similar findings were observed in an ovarian cancer cell line (Chen et al., 2009). Alternatively, it has been shown in our lab that abolishing DPP5 expression in LOX melanoma cells would result in significantly less aggressive and metastatic behavior of tumors in an animal model (Donghai Chen, unpublished data). Similarly, targeting DPP5 through genetic deletion or pharmacologic agents is shown to inhibit tumor stromagenesis and growth in a mouse model (Santos et al., 2009).

In colorectal cancer, it was shown that there was a significant correlation between DPP5 expression and lymph node involvement (Iwasa et al., 2005). Another study on primary colon cancer reported that tumor stromal DPP5 staining was higher in earlier stage tumors suggestive of a more prominent role in early development of tumors. However, greater stromal DPP5 staining in patients with known metastatic disease was associated with decreased survival (Henry et al., 2007). Another study has suggested that DPP5 expression can promote survival of primary myeloma plasma cells (Ge et al., 2006). More recently, it has been shown that increased stromal expression of DPP5 in esophageal adenocarcinoma patients is associated with worse outcome (Saadi et al., 2010).

It has been proposed that tumor-associated expression of DPP5 can be exploited as a potential diagnostic and therapeutic tool (Pure, 2009). Welt et al. (Welt et al., 1994) initially described the possible diagnostic and therapeutic use of humanized monoclonal antibodies directed against DPP5. Additional preclinical studies (Ostermann et al., 2008) and small phase 1 and phase 2 clinical trials using monoclonal antibodies against DPP5 had initial promising results (Scott et al., 2003). Administration of the small-molecule inhibitor of DPP5, Val-boro-Pro (Talabostat), resulted in minimal clinical effect in patients with previously treated metastatic colorectal cancer. However, incomplete inhibition of

DPP5 enzymatic activity in the peripheral blood was observed, and suggested as a possible explanation of the observed limited clinical effectiveness (Narra et al., 2007). In another study, DPP5-selective peptide protoxins were constructed and it was shown that their direct injection into human breast and prostate xenograft tumors in animal models resulted in significant lysis and growth inhibition of tumors with minimal toxicity to the host animal (Lebeau et al., 2009). Finally, use of DPP5-specific antibodies to coat nanoparticles containing tumor necrosis factor in order to target them specifically to DPP5-expressing tumors has been under investigation (Messerschmidt et al., 2009).

While the studies described above mostly focus on DPP5 as a membrane-bound protein, a soluble form of DPP5 has been identified in bovine plasma, which appears to be present in physiologic condition (Collins et al., 2004). More recently, presence of DPP5 in human plasma under physiologic conditions with potential role in coagulation has also been confirmed and described as antiplasmin-cleaving enzyme (APCE) (Lee et al., 2006). These findings suggest another possible mechanism for incomplete inhibition of DPP5 enzymatic activity in patients observed in clinical studies (Narra et al., 2007), since the presence of free DPP5 in the circulation is expected to compete with tumor-associated DPP5 for the inhibitor. As such, soluble DPP5 should be considered when designing DPP5-specific anti-tumor therapies.

Shedding and secretion of DPP5 from cells into a soluble form have been described. The origin of the soluble DPP5 in plasma remains unknown. It has been reported that a recombinant soluble form of DPP5 (lacking the transmembrane domain) undergoes successive N-terminus proteolytic cleavage in culture medium from the initial 160 kDa dimer to dimers of 100 to 85 kDa and 70 to 50 kDa with increased gelatinase activity (possibly as a result of decreased steric hindrance). This truncation is inhibited in the presence of EDTA, suggestive of involvement of metalloproteases (Chen et al., 2006). It is not known if a similar mechanism is involved in release of membrane-bound DPP5 into extracellular space. N-terminal amino acid sequencing has not been performed on soluble DPP5 yet, but given the position of the transmembrane domain of the protein

(amino acid 7-26), and the homology with DPP4 (see below), if proteolytic release is involved, it is likely that the cleavage occurs in or beyond the stalk region (i.e. beyond 26th amino acid).

Analysis of DPP5 amino acid sequence by the PSORT II program (available at http://psort.ims.u-tokyo.ac.jp/) confirms that DPP5 has a type II transmembrane topology with a short cytoplasmic N-terminus. However, there are no predicted signal peptides, cleavage sites for mitochondrial presequence, nuclear localization signal, endoplasmic reticulum (ER) retention motif, ER membrane retention signal, peroxisomal targeting signal, vacuolar targeting motif, RNA-binding motif, DNA-binding motif, actinin-type actin-binding motif, N-myristoylation pattern, farnesylation/geranylgeranylation motif, transport motif from cell surface to golgi, or ribosomal protein motifs. Additionally, alternative splicing of mRNA (to produce putative secretory isoforms of DPP5) does not seem to be a likely mechanism for DPP5 release either, as the identified alternatively spliced form of DPP5 mRNA lacks most of the N-terminus sequences (including the transmembrane domain) and based on sequence analysis and other data, it seems to be confined to within the cytoplasm (Goldstein and Chen, 2000). Of note, available DPP5 monoclonal antibodies do not detect this form. No alternative splicing has been reported for DPP4 mRNA (Goldstein and Chen, 2000). Based on all above, proteolytic release and shedding of DPP5 from membrane appears to be a plausible mechanism.

Unlike DPP5, DPP4 has been extensively studied. It is a multifunctional protein which (similar to DPP5) exists in both membrane-bound and soluble forms. DPP4 is constitutively expressed in many tissues and it is present on surface of most cell types as well is in most body fluids (including plasma) in relatively high concentrations (Havre et al., 2008). The plasma level of DPP4 varies in different reports, and the reported mean (or median) values, measured by enzyme-linked immunosorbent assay (ELISA) in various healthy or patient populations range from 0.1 to 16 μg/mL (with a midpoint plasma concentration of around 7 μg/mL in healthy humans; level in women being lower than men) (Gorrell et al., 2001; Cordero et al., 2009). Despite extensive research, the source

of soluble DPP4 in plasma is not fully understood. Hepatobiliary system is the most often cited tissue source, followed by immune system (e.g. shed from T lymphocytes) and spleen (Cordero et al., 2009). Proteolytic cleavage and shedding of the membrane-bound form of DPP4 from the plasma membrane of DPP4-expressing cells that are in contact with blood is the commonly accepted mechanism of its release into circulation (Iwaki-Egawa et al., 1998; Cordero et al., 2009). The proteolytic cleavage is believed to take place between the 38th and 39th amino acid of the N-terminus end of the membrane-bound form (Iwaki-Egawa et al., 1998).

DPP4 has been used as a tumor marker and its level (either the membrane-bound or plasma circulating form) has been found to be correlated with various tumor types, with varying degree of clinical significance. The membrane-bound form has been reported to be increased in certain tumors (e.g. mesotheliomas and renal carcinomas) and decreased in some other tumors (e.g. breast cancers, endometrial cancers, melanomas and oral cancers). Its level is found to be variable in colorectal, lung, prostate and ovarian carcinomas, gliomas and hematological malignancies, with mixed reports on its association with tumor invasiveness (Havre et al., 2008).

With regards to the plasma DPP4, some studies have looked at the antigenic concentration (as measured by ELISA), and some have measured the DPP activity, with the former being generally considered as the more accurate and reliable method. Overall, data indicate that low levels of plasma DPP4 are generally associated with impaired immune status, while increased levels are more often encountered in conditions associated with activation of immune system (e.g. inflammation and infections). DPP4 ELISA measurement data in cancer patients are limited, and available reports indicate increased level in oral squamous cell carcinoma (SCC), decreased levels in colorectal cancers, and unchanged levels in gastric cancers, compared with healthy individuals (Cordero et al., 2009). Data on plasma DPP4 enzymatic activity (more precisely, DPP activity) in cancer patients are more extensive, and indicate increased DPP activity in hepatocellular, biliary tract, gastric and hematologic cancers, decreased

activity in pancreatic cancer and oral SCC, and variable levels of activity in gastric cancers (Cordero et al., 2009). It has been suggested that the apparent discrepancies between the antigenic and activity measurements in some cancers may be due to altered immunorecognition as a result of glycosylation patterns, presence of activators, and presence of other molecules with DPP activity in plasma. Notably, some 10% of the DPP activity in plasma cannot be accounted for by the presence of DPP4 alone (Cordero et al., 2009). As mentioned earlier, DPP5 possesses DPP activity, and it may play a role here.

The explanation behind fluctuation of DPP4 level in plasma in various cancers is lacking. Given the evidence suggesting hepatobiliary system and immune cells as the primary physiologic sources of plasma DPP4, increased plasma DPP4 levels in tumors arising from these tissues is expected. It has been suggested that some other tumors may actively cause the soluble DPP4 level to drop (e.g. through secreting immunosuppressive factors such as TGF-β_1 which can down-regulate DPP4 expression in many immune cells) and thereby undermine the immune response against them. Conversely, higher than normal levels of soluble DPP4 has been reported in patients with advanced and metastatic cancers (Cordero et al., 2009).

To date, the source of soluble DPP5 is undetermined and there has been no study examining the clinical utility of measuring plasma level of DPP5 and to correlate that with plasma DPP4 level in patients with cancer. This study was conducted to develop immunologic assays for measurement of DPP5 and DPP4 in plasma and to assess the levels of these peptidases in patients with various forms of cancer as compared with healthy subjects, as well as their association with patient characteristics, tumor characteristics, and survival. Finally, role of proteases in releasing DPP5 from cell membrane was investigated.

SPECIFIC AIMS AND HYPOTHESES

1. Is level of soluble DPP5 in plasma correlated with age, gender and health status (healthy versus having cancer)?

Although membrane-bound DPP5 has been found in many tumors, the plasma level of the soluble form of DPP5 in healthy individuals and cancer patients and its correlation with patient characteristics are unknown.

Hypothesis: Plasma DPP5 level is different among healthy individuals and cancer patients, within various cancer types, and among male and female subjects.

Experiment: Level of DPP5 is measured by ELISA in plasma samples of healthy individuals and cancer patients and its association with age, gender, health status and cancer type is analyzed.

2. Is the level of plasma DPP5 correlated with the level of plasma DPP4?

DPP4 has been shown to be associated with DPP5 on cell surface, but it is not clear if the levels of the two in plasma are also related. Measurement of DPP4 can also provide an internal control for the specificity of the methods.

Hypothesis: Level of plasma DPP5 is correlated with the level of plasma DPP4 in healthy individuals and cancer patients.

Experiment: Level of DPP4 is measured by ELISA in same plasma samples used in Specific Aims 2 and 3, and its correlation with DPP5 levels and clinical parameters, as well as its effects on survival will be analyzed.

3. Is level of soluble DPP5 in plasma associated with tumor stage and survival of cancer patients?

Membrane-bound DPP5 in tumors has been linked to prognosis in a number of cancers, but it is not clear if the level of plasma DPP5 is associated with cancer stage and patient survival.

Hypothesis: Patients with higher level of soluble DPP5 in plasma have higher stage cancer and worse prognosis.

Experiment: Association of the level of DPP5 in plasma of cancer patients with the tumor stage as a general prognostic factor will be analyzed. Furthermore, association between plasma DPP5 level and survival will be studied.

4. Is membrane-bound DPP5 proteolytically cleaved and released as a soluble form?

DPP5 is an integral membrane protein and it lacks secretory signal peptides. The source of plasma DPP5 is not known. Evidence suggests that the other member of DPP family, DPP4, is proteolytically cleaved from cell membrane and released into blood. It has also been shown that recombinant soluble DPP5 undergoes proteolytic truncation from N-terminus and this truncation is inhibited by EDTA, suggestive of possible role of metalloproteases.

Hypothesis: Inhibition of proteases inhibits shedding of membrane-bound DPP5 into medium.

Experiment: Following establishing that DPP5 is released into culture medium from a DPP5-expressing cell line model, the presence of soluble DPP5 in serum-free media and the effect of inhibitors of major proteases on the level of soluble DPP5 in the serum-free medium conditioned by the cells will be measured using ELISA. This experiment will examine if inhibition of any group of enzymes would inhibit the release of DPP5 from membrane. Finally, the cell-conditioned medium will be ultracentrifuged to remove shed membrane vesicles and the supernatant will be tested by ELISA as a control for trapping of DPP5 in shed membrane vesicles.

MATERIALS AND METHODS

- Human Samples:

Plasma samples used in this study were procured from consenting adult volunteers under a Stony Brook University IRB-approved protocol ("Early Carcinoma Antigens" study, protocol number 20073696). Participants consisted of healthy individuals and patients newly diagnosed with various malignancies at Stony Brook University Cancer Center (Stony Brook, NY) and Veteran Administration Medical Center (Northport, NY). Venous blood for this study was collected prior to surgery and chemo/radiotherapy. A limited number of subjects had subsequent second blood-draw(s) following surgery and during chemo/radiotherapy. In each case, approximately 5 mL blood was drawn into BD green-capped Vacutainer® heparin tubes containing 76 IU lithium heparin. Samples were centrifuged at 1,000 rpm for 3 minutes; plasma was removed, aliquoted and stored at -80ºC until used. The follow-up duration 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.

Presence of DPP5 in human plasma was documented using Western blotting of boiled, cleared diluted human plasma run on SDS-PAGE. The identified bands corresponded to ~97, ~65, ~35, and ~25 KDa DPP5 monomer bands previously reported in LOX tumor tissue lysates from a xenograft model (Chen et al., 2006), but also, ~50 and ~130 KDa bands (possibly dimers of 25 and 65 KDa monomers) were observed (Figure 2).

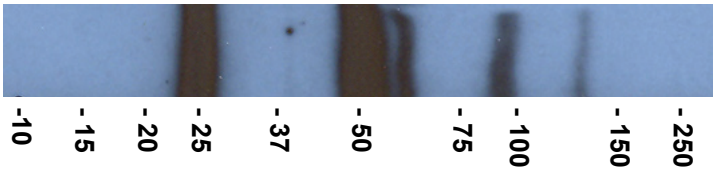

Figure 2- Western blot of DPP5 in human plasma. Boiled, cleared diluted human plasma was run on SDS-PAGE, blotted, and visualized using E97 mAb. Numbers represent marker bands (KDa).

- Production and Purification of Recombinant Soluble DPP5 and DPP4:

HEK293 cells were transfected with a modified pCEP4 vector harboring DPP5 sequence without the transmembrane domain and with an N-terminus mouse Igκ secretion signal and a C-terminus V5-His tag. Cells were maintained in complete medium (DMEM, 10% fetal bovine serum [FBS], 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin) and selected initially by adding G418 (250 μg/mL) and Hygromycin B (200 μg/mL), as previously described (Chen et al., 2006). Drug-resistant colonies were further screened for secretion of recombinant soluble DPP5 (rDPP5) into medium by an antibody-capture enzymatic assay, serially testing for DPP and gelatinase activities, as previously described (Chen et al., 2006). The colonies with highest expression of rDPP5 were identified and cultured in serum-free medium (SFM4HEK, Hyclone) to produce high levels of rDPP5. Conditioned culture medium was tested for presence of DPP5 both enzymatically (as mentioned above) and by western blot. The final medium was preserved by addition of EDTA to a final concentration of 5 mM to suppress further proteolysis of DPP5 by metalloproteases.

The conditioned culture medium was cleared, and proteins were precipitated by addition of ammonium sulfate, which were resuspended in PBS with 5mM EDTA. Following additional buffer exchange with PBS with 5 mM EDTA to remove the remaining ammonium sulfate, rDPP5 was purified using a His-tag purification column. Purity of recombinant protein was verified using SDS-PAGE and the protein was confirmed to be rDPP5 using Western immuno-blotting and antibody-capture enzymatic assays for dipeptidyl peptidase and gelatinase activities (Figure 3). The purity and concentration of rDPP5 was verified and assessed using SDS-PAGE (Figure 4). Glycerol to final volume of 50% was added to the purified rDPP5 samples and they were stored at -80ºC until used. Similar process was used to produce and purify recombinant DPP4 (rDPP4).

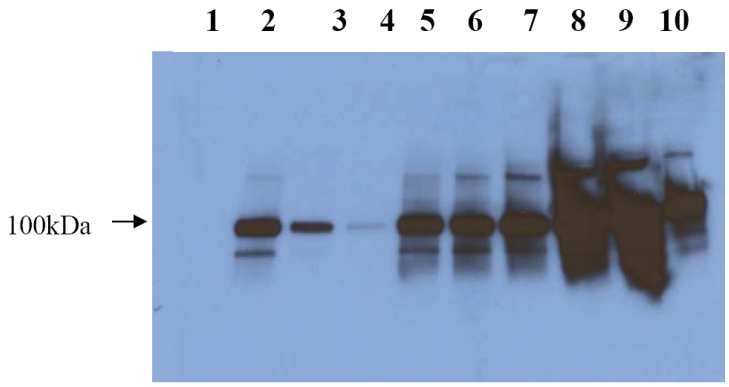

Figure 3- Purification of rDPP5. Western blot using anti-DPP5 mAb E97 is shown. The sample used was prepared from desalting ammonium sulfate precipitated serum-free medium conditioned by HEK293 cells transfected with an rDPP5-expressing vector. Lanes: 1, marker; 2, desalted sample prior to loading into His-Trap column; 3, the flow-through from His-trap column after loading the sample; 4, wash flow-through using 0.5M NaCl, 5mM imidazole in 20mM Tric-HCl pH 7.9; 5-7, subsequent wash flow-through using 0.5M NaCl, 60mM imidazole in 20mM Tric-HCl, pH 7.9; 8-10, elution using 0.5M NaCl, 1M imidazole in 20mM Tris-HCl pH 7.9. Overexposure in lanes 8-10 is indicative of rDPP5 enrichment.

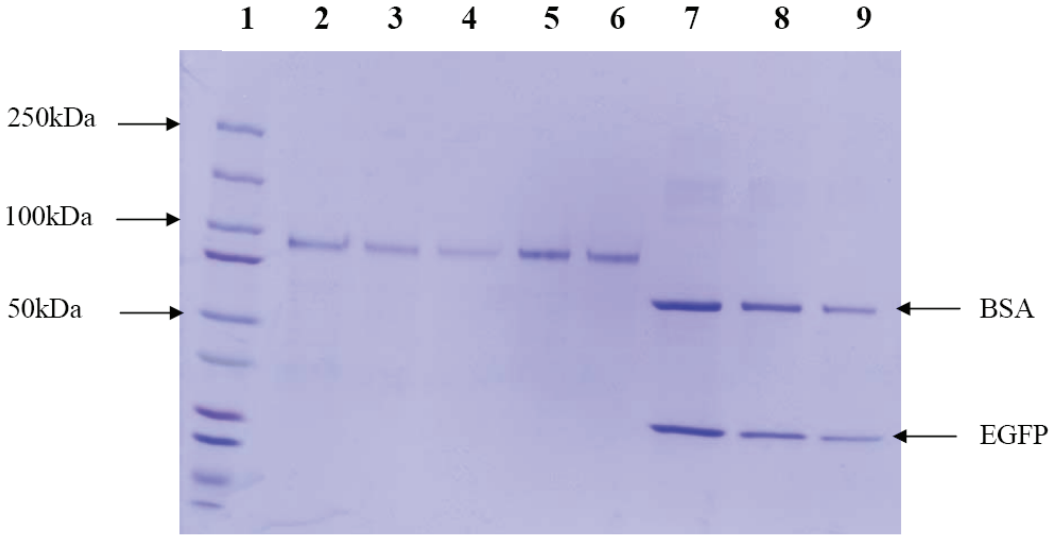

Figure 4- Purity and concentration of rDPP5. Eluted samples from His-Trap column were run on SDS-PAGE. Lanes: 1, marker; 2-6, His-Trap-purified rDPP5 samples; 7-9, BSA and EGFP standards. Based on the standards and using Imagequant TL (GE Healthcare), the concentrations of rDPP5 in the samples (lanes 2-6) were estimated to be 0.048, 0.028, 0.026, 0.068, and 0.076 mg/mL, respectively.

- DPP5 and DPP4 ELISA:

An ELISA to measure DPP5 and DPP4 in plasma was developed. Various mAb combinations (all previously validated to be specific for their respective target proteins (Chen et al., 2006)) were tested to determine the best pair to yield a sensitive and specific assay. The final ELISA reagents and protocol used in this study are as follow:

- *PBST 20X:* NaCl (160 g), KCl (4 g), Na_2HPO_4 (28.8 g), KH_2PO_4 (4.8 g), Tween 20 (10 mL) were mixed in dH20 and final volume was adjusted to 1000 mL with dH_2O.

- *Assay Buffer:* EDTA (1.86 g) and BSA (10 g) were added to PBST 1X, total volume was adjusted to 500 mL, and stored at 4°C.

- *DPP5 Standard Stock (SSS; 0.38 µg/mL):* Using purified rDPP5, batch-1 (48 µg/mL, >90% purity), 8 µL of purified rDPP5 was added to 492 µL Assay Buffer and mixed. Glycerol (500 µL) was added, mixed and stored at -20°C. Alternatively, using purified rDPP5, batch-5 (76 µg/mL, >95% purity): 5 µL of purified rDPP5 was added to 495 µL Assay Buffer and mixed. Glycerol (500 µL) was added, mixed and store at -20°C.

- *DPP4 Standard Stock (DSS; 3.84 µg/mL):* Using purified rDPP4, batch 2 (24 µg/mL, >99% purity), 160 µL of purified rDPP4 was added to 340 µL Assay Buffer and mixed. Glycerol (500 µL) was added, mixed and stored at -20°C.

- *ELISA Protocol:* The protocol was optimized to simultaneously measure DPP4 and DPP5 from up to 8 different plasma samples in triplicate on each single plate. Microtiter U-bottom 96-well plates (Dynex Technologies) were used. Template for the ELISA plate is depicted in Figure 5. The following steps were performed:

1. The 96-well plate was coated with 100 µL of 10 µg/mL purified rat anti-human-DPP5 mAb (D28) or rat anti-human-DPP4 mAb (E26) in PBS overnight at 4°C.

2. The plate was emptied and washed 3 times with PBST.

3. The plate was blocked with addition of 200 µL of Assay Buffer, and incubated for 2 hours at ambient temperature.

4. For each plate…

- Samples (n=8, 0.1% plasma):
    i. Each sample was completely thawed and mixed.
    ii. 20 µL of each sample was added to 980 µL Assay Buffer and mixed (2% plasma).
    iii. 16 microtubes (8 for DPP5, 8 for DPP4; 2 for each sample) were labeled and 180 µL Assay Buffer was added to each.
    iv. 20 µL of each 2% sample was added to the corresponding labeled microtube and mixed (0.2% plasma).
    v. The remaining 2% plasma samples were saved and stored at -20°C for future use.
- DPP5 standards (n=8; from 3.8 ng/mL to 60 pg/mL and 0):
    i. 8 µL of SSS was added to 392 µL Assay Buffer and mixed (S1).
    ii. 200 µL Assay Buffer was added to 7 other microtubes (S2-S8).
    iii. Two-fold serial dilutions were made by transferring 200 µL from S1 to S2 down to S7, each step with adequate mixing; the last 200 µL was discarded from S7.
    iv. Nothing was added to Assay Buffer in S8 (negative control).

- DPP4 standards (n=8; from 38.4 ng/mL to 0.6 ng/mL and 0):
    i. 8 µL of SSD was added to 392 µL Assay Buffer and mix (D1).
    ii. 200 µL Assay Buffer was added to 7 other microtubes (D2-D8).
    iii. Two-fold serial dilutions were made by transferring 200 µL from D1 to D2 down to D7, each step with adequate mixing; the last 200 µL was discarded from D7.
    iv. Nothing was added to Assay Buffer in D8 (negative control).

5. 3.5 mL of the 2 µg/mL digoxigenin-conjugated rat anti-human-DPP5 mAb D8 and 3.5 mL of digoxigenin-conjugated rat anti-human-DPP4 mAb E3 in Assay Buffer were prepared. 200 µL of D8 solution was added to each DPP5 microtube; 200 µL of E3 solution was added to each DPP4 microtube and mixed (total volume of each microtube = 0.4 mL).
6. Microtubes were incubated for 1 hour on a shaker at ambient temperature.
7. The Assay Buffer was discarded from the plate and it was washed 3 times with PBST.
8. From each microtube, 300 µL was transferred to 3 adjacent wells (100 µL per well) as per the plate template (Figure 5).
9. The plate was incubated for 1 hour on a shaker at ambient temperature.
10. The wells' contents were discarded and the wells were washed 5 times with PBST.
11. 11 mL of 50 mU/mL anti-Digoxigenin-POD poly Fab fragment (Roche) in Assay Buffer was prepared; 100 µL was added to each well.
12. The plate was incubated for 1 hour on a shaker at ambient temperature.

13. 11 mL of TMB liquid substrate for ELISA (Sigma) was poured into a tube, covered with foil and left at room temperature to equilibrate with ambient temperature.

14. The wells' contents were discarded and the wells were washed 10 times with PBST.

15. 100 µL of TMB substrate was added to each well, and the color reaction was monitored under dimmed light (usually for ~4 minutes); this step was not done on a shaker.

16. The reaction was stopped by adding 100 µL of 2N HCl to each well. The wells were mixed and any air bubbles were removed.

17. The plate was read using a Microplate Spectrophotometer System (Molecular Devices), at 450 and 570 nm, and the optical density (absorbance) at 570 nm was subtracted from 450 readings.

	1 2 3	4 5 6	7 8 9	10 11 12
A	Sample 1 (0.1%)	Sample 1 (0.1%)	Sample 2 (0.1%)	Sample 2 (0.1%)
B	Sample 3 (0.1%)	Sample 3 (0.1%)	Sample 4 (0.1%)	Sample 4 (0.1%)
C	Sample 5 (0.1%)	Sample 5 (0.1%)	Sample 6 (0.1%)	Sample 6 (0.1%)
D	Sample 7 (0.1%)	Sample 7 (0.1%)	Sample 8 (0.1%)	Sample 8 (0.1%)
E	S1 (3.8 ng/mL)	D1 (38.4 ng/mL)	S2 (1.9 ng/mL)	D2 (19.2 ng/mL)
F	S3 (0.95 ng/mL)	D3 (9.6 ng/mL)	S4 (0.48 ng/mL)	D4 (4.8 ng/mL)
G	S5 (0.24 ng/mL)	D5 (2.4 ng/mL)	S6 (0.12 ng/mL)	D6 (1.2 ng/mL)
H	S7 (0.06 ng/mL)	D7 (0.6 ng/mL)	S8 (0 ng/mL)	D8 (0 ng/mL)
	Coated withD28 (DPP5 mAb)	Coated with E26 (DPP4 mAb)	Coated with D28 (DPP5 mAb)	Coated with E26 (DPP4 mAb)

Figure 5- ELISA plate template. Microtiter U-bottom 96-well plates (Dynex Technologies) were used.

Results of each sample were considered acceptable if they met the following criteria:

- Coefficient of Variation (CV) among the triplicates less than 10%;
- Measurement within the linear standard range; and
- R^2 of the standard curve regression line of the plate at least 0.95

CV was used as a normalized measure of dispersion of the individual readings in triplicate wells and it was calculated by dividing the standard deviation (SD) of the individual readings in a triplicate by the mean of the readings multiplied by 100.

The performance of the developed DPP5 and DPP4 ELISAs was verified. Using serial dilutions of recombinant proteins, the lowest detection level of DPP5 assay was estimated to be 60 pg/mL. At this concentration, assay reading was greater than negative control by 2 SD in 90% of measurements. Given this observation and linear range of the measurements, DPP5 standards for the assays were prepared to be in the range of 0.06-1.9 µg/mL (Figure 6). Likewise, the lowest detection limit of DPP4 assay was estimated to be at least 600 pg/mL. At this concentration, assay reading was greater than negative control by 2 SD in 100% of measurements. The DPP4 standards were prepared to be in the range of 0.6-19.2 µg/mL (Figure 7). Any readings not falling in these ranges were considered to be unacceptable and assays were repeated at a different dilution.

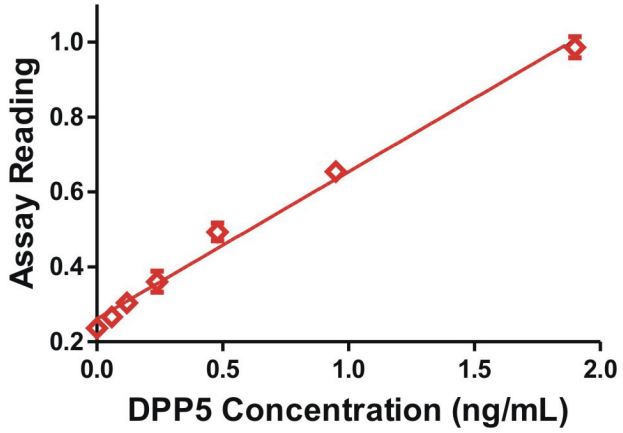

Figure 6- DPP5 ELISA standard curve. Assay reading (in terms of optical density [OD] at 450 nm minus 570 nm) using serial dilutions of rDPP5. Error bars represent SD.

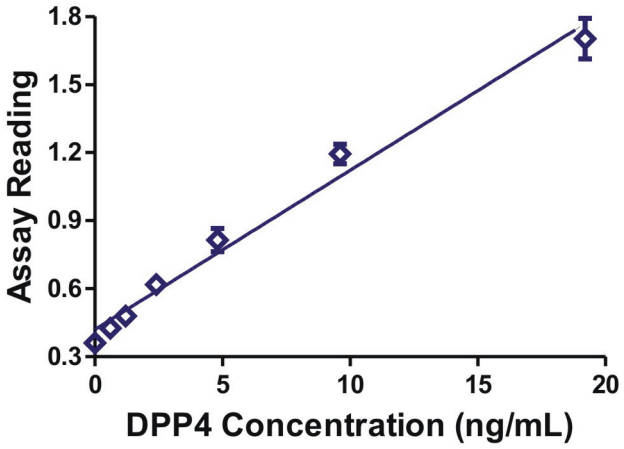

Figure 7- DPP4 ELISA standard curve. Assay reading (in terms of optical density [OD] at 450 nm minus 570 nm) using serial dilutions of rDPP4. Error bars represent SD.

The specificity of the monoclonal antibodies used in developing these assays has been previously documented (Chen et al., 2006). Furthermore, the assays were specific for the target protein as indicated by the following:

1) The readings of the DPP5 assay were indistinguishable from the negative control when various concentrations of purified rDPP4 were used as the sample. Likewise, the DPP4 assay did not show any cross-reactivity with rDPP5;
2) The assays showed no cross-reactivity with bovine DPP5 and DPP4 (as tested with various concentrations of bovine serum, previously reported to contain DPP5 and DPP4 (Collins et al., 2004)); and
3) The assays 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 (DPP4 or DPP5) by using antibody-coated beads.

Following addition of a known amount of rDPP5 to plasma samples, on average, 90.7% of the spiked rDPP5 was recovered in the assay, and the measured DPP4 level changed only 0.6%. Conversely, following addition of a known amount of rDPP4 to plasma samples, only 51.8% of the spiked r-DPP4 was recovered in the assay. The measured DPP5 changed only 3.8% (Figure 8).

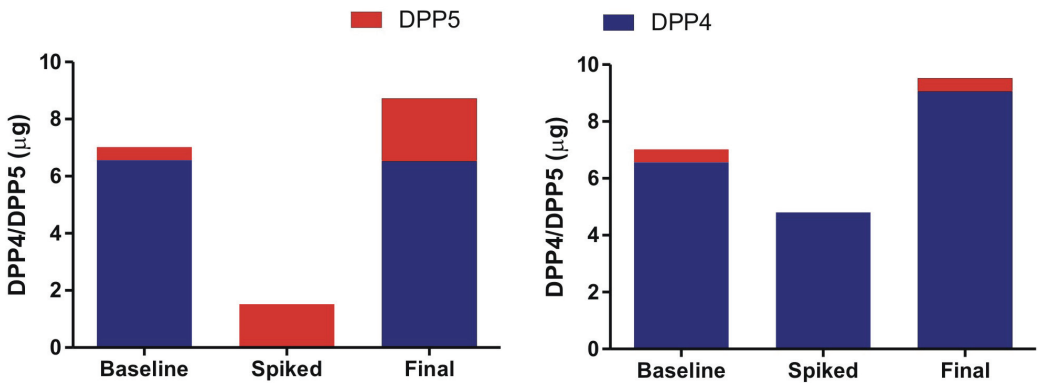

Figure 8- rDPP4/rDPP5 spiking in plasma. Levels of DPP4 and DPP5 in human plasma samples were measured using ELISA ("Baseline") and known amounts of r-DPP4 or r-DPP5 were added to plasma samples ("Spiked"). The new levels of DPP4 and DPP5 following addition of recombinant proteins were measured using ELISA ("Final"). Values are presented by the height (or area) of the bars.

- Proteolytic Release of Membrane-bound DPP5:

Human tumor cell lines that natively express membrane-bound DPP5 were cultured in regular as well as serum-free medium and presence of DPP5 in their conditioned medium was assessed using ELISA. Initial tests revealed that DPP5 was present in the conditioned medium of LOX melanoma cell line (known to constitutively express high levels of membrane-bound DPP5 (Goldstein et al., 1997; Pineiro-Sanchez et al., 1997)). The subsequent experiments focused on LOX cell line, as well as DPP5 knocked-down LOX variants previously created (Chen et al., 2006).

LOX cells were cultured to near confluence in regular medium, and viability was confirmed to be near 100% using Trypan blue dye exclusion test. The conditioned medium was used to test whether the DPP5 is free in medium, or associated with membranes (e.g. vesicles shed from the cells). Conditioned medium (20 mL) was filtered and ultracentrifuged at 105,000 × g at 4°C for 90 minutes to pellet the vesicles as previously described (Taverna et al., 2003). The unconditioned medium, unprocessed conditioned medium, supernatant, as well as the pellet (resuspended in 0.5 mL unconditioned medium with 1% n-octyl p-D-glucopyranoside [OG]) were used for ELISA measurement of DPP5. In all

experiments and based on previous reports (Chen et al., 2006), EDTA to a final concentration of 5 mmol/L was added to samples immediately after removal from cell cultures to inhibit further proteolytic truncation of DPP5.

Table 1- Protease inhibitors. Various Protease inhibitors were studied for their effect on release of DPP5 from cells into culture medium. Inhibitors (Sigma product code INHIB1) against four group of proteases (Aspartyl proteases, Cysteine proteases, Metalloproteases, and Serine proteases) were utilized (Ghersi et al., 2002; Ghersi et al., 2006; Chen et al., 1984). For each inhibitor, concentrated solution was prepared in the appropriate solvent and added to respective wells (1mL total volume per well) to acheive the indicated final concetration. Same volumes of various solvents without inhibitors were used as controls.

Inhibitor Name	Target of Inhibitor Activity	Final Concentration	Solvent Used	Cell Toxicity
α_2-Macroglobulin	Ser/Cys/Asp/Metalloproteases	0.22 mg/mL	PBS	None
AEBSF	Serine protease	0.06 mg/mL	PBS	None
Antipain	Serine/Cysteine and some trypsin-like serine proteases	0.25 mg/mL	PBS	None
Aprotinin	Serine proteases	0.2 mg/mL	PBS	None
Benzamidine	Trypsin; Serine proteases	0.31 mg/mL	PBS	None
Bestatin	Leucine aminopeptidase, aminopeptidase B and triamino peptidase	13.8 µg/mL	PBS	None
Chymostatin	Wide-range	0.2 mg/mL	DMSO	None
E64	Cysteine proteases	36 µg/mL	PBS	None
EACA	Chymotrypsin, FVIIa, Lysine carboxypeptidase, plasmin, plasminogen activator	0.5 mg/mL	PBS	None
EDTA	Metalloproteases	1 mM	DMEM	None
GM6001	MMPs	9.7 µg/mL	DMSO	None
Leupeptin	Ser/Cysteine proteases	0.24 mg/mL	PBS	None
NEM	Cystein proteases	0.125 mg/mL	Ethanol	None
Pepstatin	Aspartyl proteases	20.6 µg/mL	Ethanol	None
Phosphoramidon	Bacterial metalloproteases	5.4 µg/mL	PBS	None
Trypsin inhibitor (Soybean)	Trypsin, chymotrypsin/plasmin	0.1 mg/mL	PBS	None

To study the effect of various protease inhibitors on release of DPP5 from cells, LOX cells were cultured in serum-free DMEM medium to near confluence, washed and resuspended using trypsin or PBS (without trypsin). In experiments using trypsin, complete medium was added to stop trypsin action. Cells were washed 3 times using serum-free medium. Near-100% viability was confirmed in an aliquot of the resuspended cells using Trypan blue dye exclusion test. Suspended cells were transferred to 24-well plates (10,000 cells in total volume of 1 mL per well), and various protease inhibitors (Sigma; Table 1) were immediately added to the cells. The cells were placed in incubator and conditioned medium was sampled from each well at 3 and 6 hours and tested for the level of DPP5 using ELISA. Following second sampling of the medium, all wells were assessed for cell viability using Trypan blue exclusion test. Immediately after each sampling of conditioned medium, EDTA (dissolved in serum-free DMEM) was added to each sample to give a final concentration of 5 mM, and the samples were cleared by centrifugation at 4000 × g at 4°C for 15 minutes and stored at 4°C until assayed by ELISA (within 24 hours).

- Statistical Analysis

DPP4 and DPP5 concentration data were verified by Kolmogorov-Smirnov and Shapiro-Wilk tests of normality to follow a normal distribution; logarithmic transformation was applied when necessary, and/or non-parametric tests were used when appropriate. Data are expressed as mean±SD or median (followed by interquartile range) unless otherwise specified. Student's t-test was used to compare the DPP4 and DPP5 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 DPP5 levels among different cancer types, different TNM scores and stages.

To assess the accuracy of plasma DPP4 and DPP5 levels in discriminating between cancer patients and healthy subjects, receiver-operative characteristics (ROC) curves were plotted, and the area under the curve was calculated. The

following ranges were used to classify the accuracy of the test based on the area under the ROC curve: 0.50 to 0.75 = fair, 0.75 to 0.92 = good, 0.92 to 0.97 = very good, and 0.97 to 1.00 = excellent (based on Simon S, Stat: ROC curve. 2000. Available at: http://www.childrens-mercy.org/stats/ask/roc.asp). The DPP4 and DPP5 cut-off points which would result in highest mutual sensitivity and specificity were determined.

Simple bivariate Pearson or Spearman's Rho tests were used as appropriate to study the correlation between DPP4, DPP5 and continuous variables following visual examination of scatter plots to rule out outliers and specific patterns. Natural logarithmic transformation was used to adjust the distribution if the normality assumption was not met and if still unsatisfactory, non-parametric variants of the above mentioned tests (i.e. Mann-Whitney U-test and Kruskal-Wallis H-test) were used as needed.

The overall effect of DPP4 and DPP5 levels on survival was studied using Cox regression. Kaplan-Meier analysis with Log Rank test was also used to study the association between DPP4 and DPP5 levels and survival, comparing groups created by cut-off points based on median DPP4 and DPP5 levels (i.e. patients with DPP5 levels above median versus below median). Whenever a statistically significant association was noted, *post hoc* analysis was performed to identify the most significant cut-off points. Analysis was 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, Illinois). Results were considered to be statistically significant if the p value was less than 0.05, except for the protease inhibitor experiments in which, due to the limited sample size and to reduce type-I error, $p<0.01$ and $p<0.001$ were used as statistical significance criteria. Assuming an alpha of 0.05, 17 samples per arm were sufficient to detect a difference of one standard deviation in DPP5 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.

RESULTS

- Assay Performance:

In total, 795 assays for DPP5 and 795 assays for DPP4 were performed on 747 human plasma samples. Overall, 18 DPP5 measurements and 20 DPP4 measurements were repeated due to not meeting the acceptance criteria (most commonly the readings were outside of the linear standard range and required adjusted dilution). Mean intra-assay CVs for DPP5 and DPP4 ELISA were below 5%. The mean inter-assay CVs for DPP5 and DPP4 ELISA were below 15% in independently performed assays (Figure 9).

DPP enzymatic assays were performed on a number of randomly-selected cancer samples, using D28 mAb (to capture and assess DPP5 activity) or E19 mAb (to capture and assess DPP4 activity) as previously described (Chen et al., 2006). The DPP enzymatic activity of the samples were found to be correlated with ELISA measurements (R^2=0.355, p=0.041 for DPP5 and R^2=0.902, p<0.001 for DPP4; Figures 10 and 11).

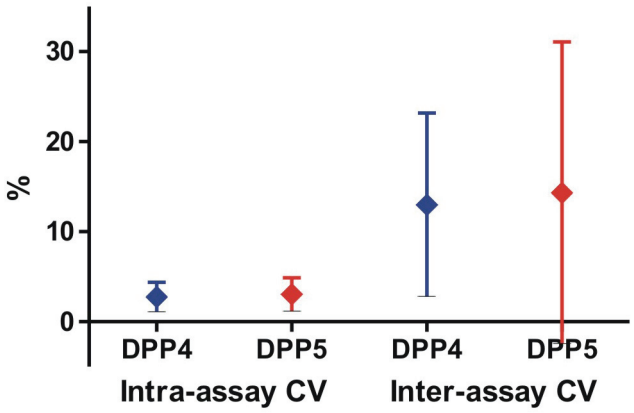

Figure 9- Intra- and inter-assay CV for DPP5 and DPP5 ELISA. Intra-assay CV was the mean of individual CVs among each triplicate for all performed assays. Inter-assay CV was the mean of CVs among repeated assays. Error bars represent SD.

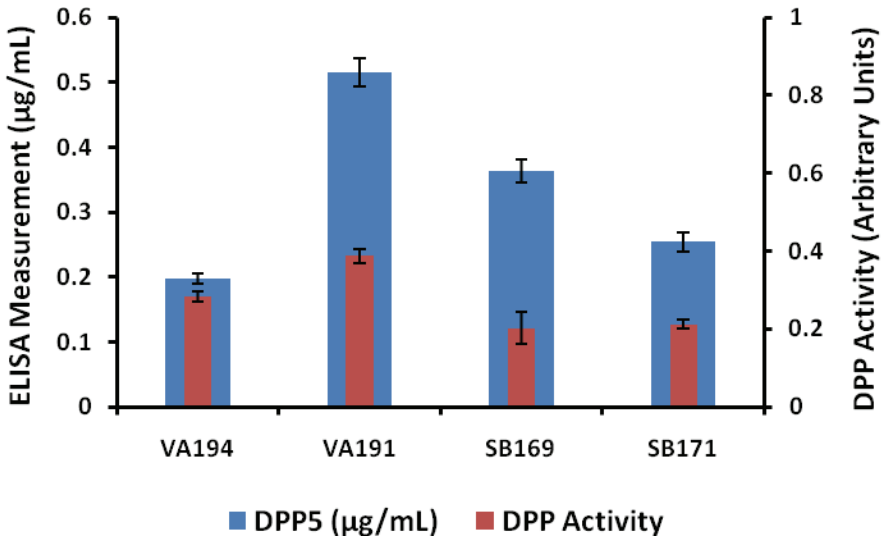

Figure 10- DPP activity of DPP5 versus ELISA measurements. DPP5 was captured from 4 plasma samples from cancer patients using D28 mAb and assayed using the Gly-Pro-nitroanilide substrate as previously described (Chen et al., 2006). P=0.041 for correlation of ELISA measurements and DPP activity. Error bars represent SD of triplicates.

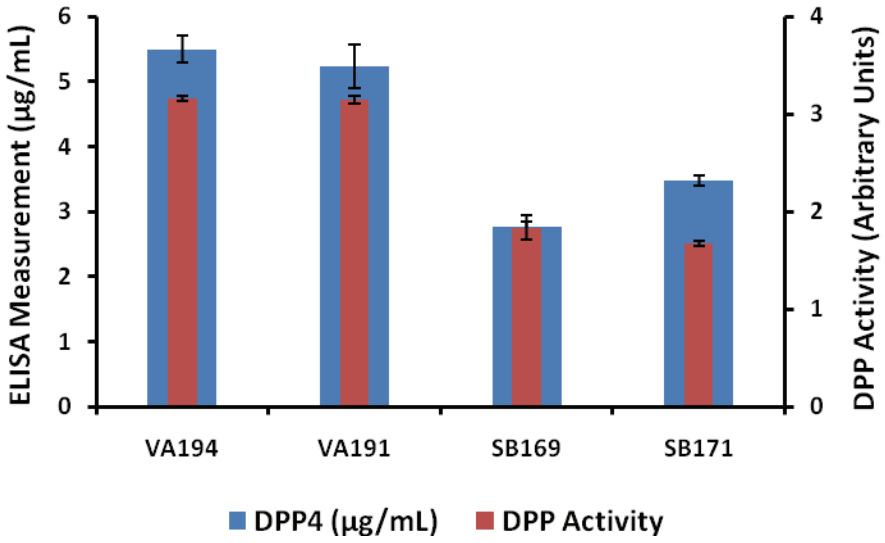

Figure 11- DPP activity of DPP4 versus ELISA measurements. DPP4 was captured from 4 plasma samples from cancer patients using E19 mAb and assayed using the Gly-Pro-nitroanilide substrate as previously described (Chen et al., 2006). P<0.001 for correlation of ELISA measurements and DPP activity. Error bars represent SD of triplicates.

- Plasma DPP5 and DPP4 Level in Clinical Samples (Specific Aims 1 and 2):

A total of 747 plasma samples were obtained from 700 subjects: 656 subjects had 1 sample and 44 had 2 samples or more obtained at different visits. The following are based on 700 plasma samples obtained on first visits, unless otherwise indicated. The distribution of the samples (healthy and cancers), patient demographics, and mean plasma DPP4 and DPP5 concentrations are presented in Table 2.

In the 747 plasma samples studied (cancer and healthy combined), mean DPP5 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 independent measurements. To ensure that these two outlier cases were not unduly affecting the statistical significance of the results, analyses were performed with and without them and the results were compared, and they were excluded from analysis as appropriate (mean DPP4 level after exclusion of these 2 cases was 4.33±2.24 μg/mL; mean DPP5 level did not change).

The DPP4 and DPP5 levels were highly correlated with each other, but several outlier cases were present as well (R^2=0.382, $p<0.001$; Figure 12). Levels of DPP4 and DPP5 were slightly higher in male 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 DPP5) (Figures 13 and 14).

In healthy subjects (Table 2), there was no significant association between age and DPP4/DPP5 levels. DPP5 was significantly higher in male subjects compared with female subjects (0.76±0.18 versus 0.65±0.18 μg/mL, p=0.015). Similarly, DPP4 was higher in male healthy subjects (6.31±1.21 μg/mL in males versus 5.65±1.48 μg/mL in female, p=0.039).

Table 2- Patient characteristics and plasma levels of DPP4 and DPP5. Values are expressed as mean±SD, except for tumor stage which is expressed in median (interquartile range [IQR]). a) Only lymphomas; b) Gynecologic malignancies included 2 cases of Clear Cell Adenocarcinoma with DPP4 levels of 109.66 and 121.99 µg/mL based on repeated measurements. Mean DPP4 of gynecologic cancer cases after exclusion of these two cases was 2.81±1.44, but mean DPP5 level stayed unchanged; c) Other/Unknown cancers included 16 urologic (other than prostate) cancers, 5 skin cancers, 1 brain cancer, 13 metastatic/secondary tumors with unknown origin, and 27 cases flagged as "cancer" but with insufficient clinical data available to determine the cancer type. GI, gastrointestinal.

Subject Category (n)	Male Subjects %	Age (years)	Metastatic Cases (%)	Tumor Stage	DPP4 (µg/mL)	DPP5 (µg/mL)
Healthy (139)	18%	43±14	-	-	5.69±1.47	0.66±0.19
Breast cancer (56)	0%	52±13	17.8%	2 (2)	4.98±1.94	0.60±0.21
Hematological cancer (28)	33%	63±10	57.1%	4 (2)[a]	3.90±2.46	0.39±0.31
Head and Neck cancer (55)	80%	67±12	66.7%	4 (0)	3.87±1.50	0.45±0.22
Colorectal cancer (88)	52%	63±12	69.8%	4 (0)	3.87±1.53	0.55±0.28
Lung cancer (100)	44%	65±11	71.4%	4 (1)	3.71±1.64	0.45±0.47
Prostate cancer (45)	100%	75±8	86.7%	4 (0)	5.70±4.41	0.54±0.20
Gynecologic cancer (100)	0%	63±12	20.2%	3 (1)	5.07±15.99[b]	0.33±0.20
Upper GI, Liver, and Pancreas cancer (27)	33%	72±9	91.7%	4 (0)	4.02±2.47	0.47±0.23
Other/unknown cancer (62)[c]	36%	69±9	Unknown	Unknown	4.51±2.39	0.54±0.29
Total (700)	27%	59±16	52.4%	4 (1)	4.65±6.37	0.51±0.30

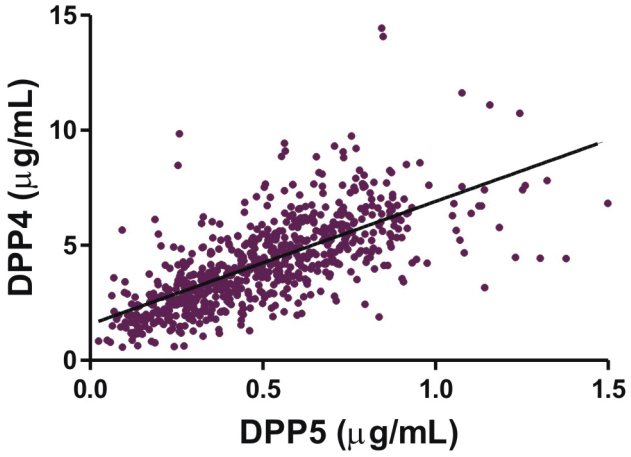

Figure 12- Association of plasma DPP4 and DPP5 levels. Scatter plot of plasma DPP4 versus DPP5 levels measured in the study population (n=692; 8 data points falling out of axis ranges were excluded). P<0.001.

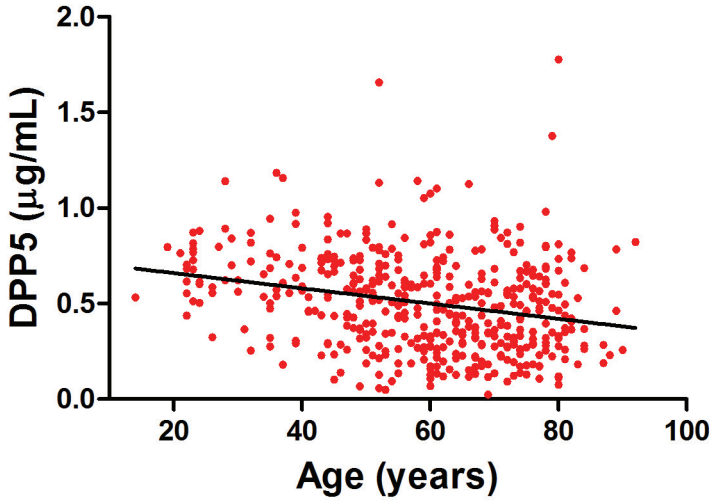

Figure 13- Plasma DPP5 level and age. Scatter plot of plasma DPP5 level versus age of the donors in all samples combined (healthy and cancer patients). P<0.001.

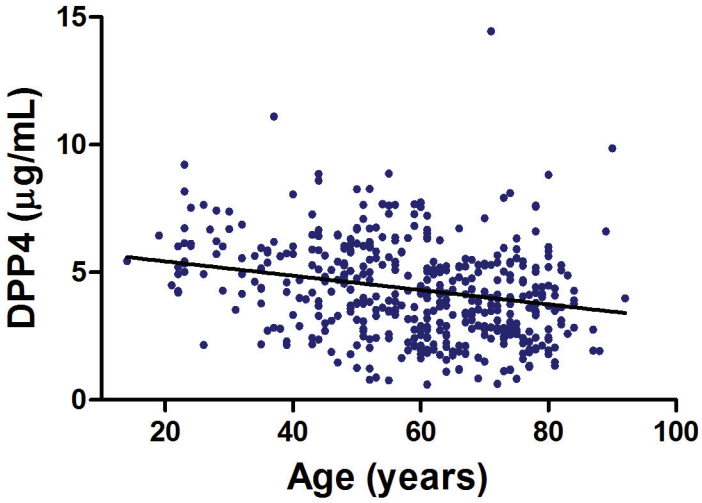

Figure 14- Plasma DPP4 level and age. Scatter plot of plasma DPP4 level versus age of the donors in all samples (healthy and cancer patients). P<0.001.

Plasma DPP5 and DPP4 levels were significantly lower in cancer patients compared with healthy subjects; the difference was more pronounced for DPP5 (3.95±2.29 µg/mL versus 5.69±1.47µg/mL, respectively, p<0.001 for DPP4; 0.47±0.30 versus 0.66±0.19 µg/mL, respectively, p<0.001 for DPP5). 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 rule out the potential effect of age on DPP4/DPP5 levels in cancer patients and healthy subjects, 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 age-matched cancer patients, p=0.285). Plasma DPP5 and DPP4 levels were again significantly lower in cancer patients compared with healthy subjects (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 DPP5).

ROC curves for DPP4 and DPP5 levels in cancer patients versus healthy subjects are presented in Figures 15 and 16. The accuracy of plasma DPP4 level to discriminate between the cancer patients and the healthy subjects was judged to be "good" (area under the ROC curve 0.788, 95% CI 0.749-0.826, p<0.001),

indicating that on average, a healthy subject had a plasma DPP4 level higher than 78.8% of the cancer patients. The maximum sensitivity and specificity to discriminate between the two groups was achieved at the DPP4 cut-off point of 4.82 µg/mL (sensitivity = 0.75, specificity = 0.75; Figure 15). The accuracy of the test increased following exclusion of stage-IV cancer cases from the ROC analysis (area under the curve 0.812, 95% CI 0.758-0.865, $p<0.001$).

Likewise, the accuracy of Plasma DPP5 level to discriminate between the cancer patients and the healthy subjects was determined to be "good" (area under the ROC curve 0.756, 95% CI 0.716-0.795, $p<0.001$), indicating that on average, a healthy subject had a plasma DPP5 level higher than 75.6% of the cancer patients. The maximum sensitivity and specificity to discriminate between the two groups was achieved at the DPP5 cut-off point of 0.50 µg/mL (sensitivity = 0.83, specificity = 0.61; Figure 16). As in the case of DPP4, the accuracy of the test increased following exclusion of stage-IV cancer cases from the ROC analysis (area under the curve 0.797, 95% CI 0.744-0.849, $p<0.001$).

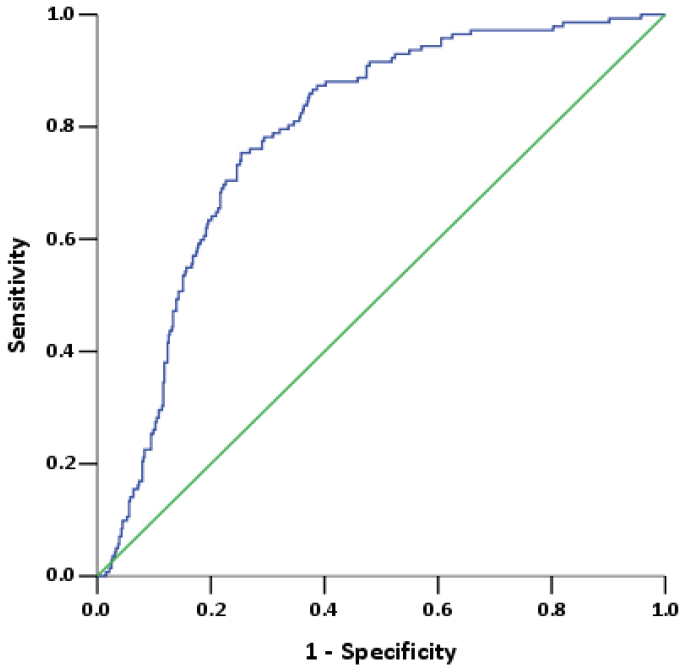

Figure 15- ROC curve of DPP4 in cancer patients versus healthy subjects. The blue curve represents the sensitivity and specificity of plasma DPP4 level to discriminate between cancer patients and healthy subjects. The closer the blue line is to the left-hand and top borders, the larger the area under the curve and the more accurate the test is.

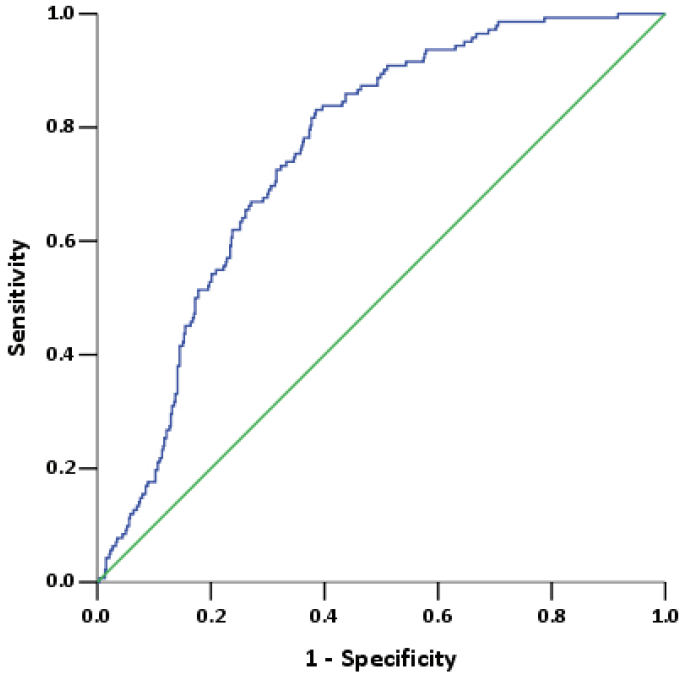

Figure 16- ROC curve of DPP5 in cancer patients versus healthy subjects. The blue curve represents the sensitivity and specificity of plasma DPP5 level to discriminate between cancer patients and healthy subjects. The closer the blue line is to the left-hand and top borders, the larger the area under the curve and the more accurate the test is.

In multiple comparisons, DPP5 levels in normal subjects were significantly higher compared with patients with gynecologic cancers ($p<0.001$), hematologic cancers ($p<0.001$), head and neck cancers ($p<0.001$), lung cancers ($p<0.001$), upper GI cancers ($p<0.001$), kidney and bladder cancers ($p=0.018$), and colorectal cancers ($p=0.030$) (Table 2). DPP4 levels in healthy subjects were significantly higher than patients with gynecologic cancers ($p<0.001$), hematologic cancers ($p<0.001$), head and neck cancers ($p<0.001$), lung cancers ($p<0.001$), colorectal cancers ($p<0.001$), and upper GI cancers ($p<0.001$). However, DPP4 and DPP5 levels were not significantly higher in normal subjects compared with patients with breast or prostate cancer.

Plasma DPP4 and DPP5 levels were significantly higher in squamous cell carcinomas (SCC) compared with adenocarcinomas. However, the former was dominated by the head and neck cancers and the latter was dominated by gynecologic and lung cancers, possibly influencing the results (Table 3).

Table 3- Plasma DPP4 and DPP5 levels in SCC versus adenocarcinomas. Gynecological cancers were uterus or ovarian. All head and neck adenocarcinomas were thyroid tumors. The reported p values are from T-test.

Cancer Site	Squamous Cell Carcinoma			Adenocarcinoma			P Value	
	N	DPP4	DPP5	N	DPP4	DPP5	DPP4	DPP5
Gynecologic	0	-	-	77	2.64±1.21	0.31±0.15	-	-
Head & Neck	25	3.98±1.72	0.46±0.22	5	4.28±0.63	0.48±0.05	-	-
Lung	1	6.03	0.35	19	3.19±1.41	0.35±0.24	-	-
Total	26	4.05±1.73	0.45±0.22	101	2.83±1.28	0.33±0.22	0.002	0.010

- Correlation of Plasma DPP5 and DPP4 Levels with Cancer Stage (Specific Aim 3):

When all cancer types were considered, there was a near significant trend toward higher DPP5 and DPP4 levels in metastatic cases: DPP4 levels in cancer patients of all types with and without metastasis at time of blood draw were 4.26±2.99 and 3.68±1.92 μg/mL, respectively (p=0.101). DPP5 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 association with lymph node involvement. There was no significant difference in DPP5 levels among cancer patients with different tumor sizes. However, DPP4 levels were significantly lower in patients with T3 compared with T1 tumors (2.78±1.18 versus 4.23±1.64 μg/mL, respectively; $p<0.001$).

DPP4 level was significantly lower in stage III cancers compared with stage I ($p<0.001$), II ($p<0.001$) and IV ($p<0.001$) stages (Figure 17). Likewise, DPP5 levels were significantly lower in stage III tumors compared with stage II ($p=0.017$) and IV ($p=0.002$) cancers (Figure 18). Table 4 summarizes the mean DPP5 and DPP4 levels in various cancer types with adequate sample size.

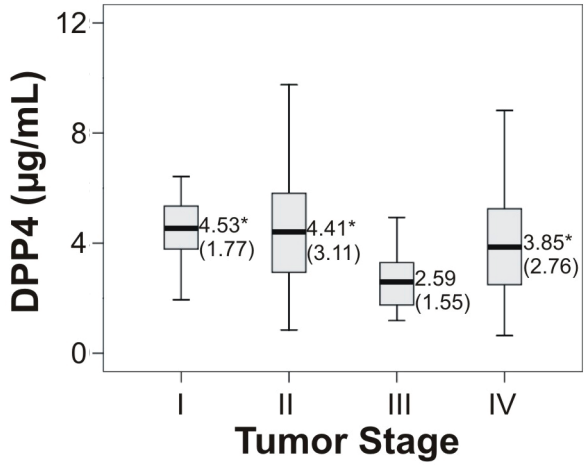

Figure 17- Plasma DPP4 level and cancer stage. Box plot of plasma DPP4 levels in cancers with different stages. Numbers represent median (interquartile range). "*" denotes p<0.001 for comparison of DPP4 levels in Stage III versus Stage II and IV.

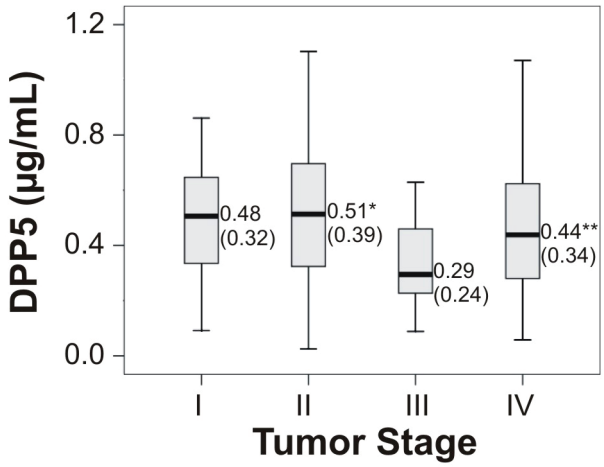

Figure 18- Plasma DPP5 level and cancer stage age. Box plot of plasma DPP5 levels in cancers with different stages. Numbers represent median (interquartile range). "*" denotes p=0.017 for comparison of DPP5 levels between Stage III and II, and "**" denotes p=0.002 for comparison of DPP5 levels between Stage III and IV.

Table 4- Plasma DPP5 levels in various stages according to cancer type. Only the cancers with adequate sample size are presented. The reported p values are from Kruskal-Wallis H-test comparing all stages to test if anyone is significantly different from others.

Cancer Site	DPP5 Concentration (µg/mL)				P Value
	Stage I	Stage II	Stage III	Stage IV	
Breast	0.60±0.13 (n=11)	0.64±0.21 (n=21)	0.54±0.06 (n=6)	0.50±0.26 (n=7)	0.302
Gynecologic	0.21±0.11 (n=8)	0.32±0.11 (n=8)	0.31±0.13 (n=44)	0.32±0.19 (n=21)	0.237
Lower GI	0.67 (n=1)	0.40±0.29 (n=6)	0.55±0.29 (n=4)	0.57±0.26 (n=40)	0.383
Lung	0.62±0.20 (n=4)	0.34±0.40 (n=3)	0.27±0.14 (n=12)	0.52±0.62 (n=51)	0.071
Prostate	0.60 (n=1)	0.70±0.09 (n=3)	- (n=0)	0.52±0.20 (n=26)	0.172
Total	0.48±0.22 (n=27)	0.52±0.26 (n=43)	0.34±0.16 (n=67)	0.50±0.40 (n=174)	<0.001

- Reproducibility of Plasma DPP5 and DPP4 Measurements:

In 44 cancer patients, a second blood sample was taken after patients underwent surgery and often during the course of chemotherapy or radiation therapy. The median time elapsed between 1st and 2nd blood sampling was 7 (5) days. DPP4 and DPP5 measurements were highly correlated between the first and second specimens obtained from each patient (R^2=0.684, p<0.001 for DPP4 [Figure 19], and R^2=0.332, p<0.001 for DPP5 [Figure 20]).

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 DPP5 measurements (p=0.720 and p=0.772, respectively) (Figures 21 and 22).

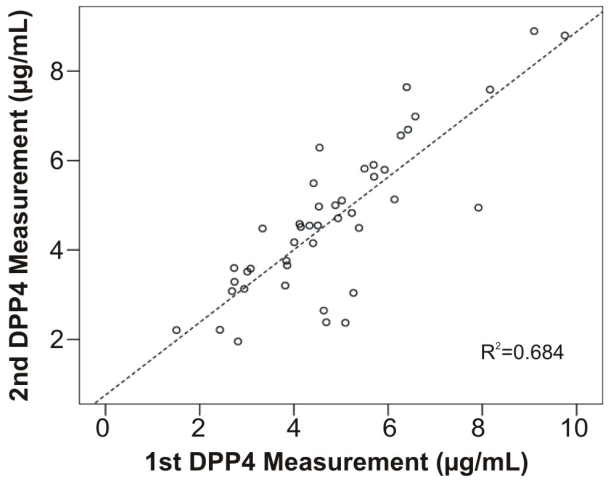

Figure 19- Serial plasma DPP4 levels in cancer patients. Scatter plot of DPP4 levels measured in 2 serial blood samples obtained from 44 cancer patients. Linear regression line is plotted.

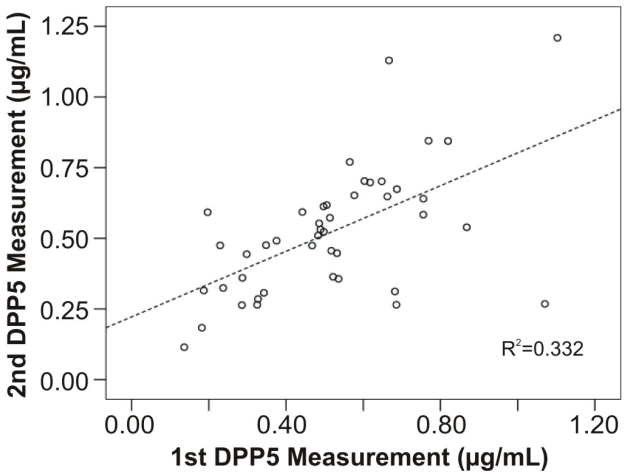

Figure 20- Serial plasma DPP5 levels in cancer patients. Scatter plot of DPP5 levels measured in 2 serial blood samples obtained from 44 cancer patients. Linear regression line is plotted.

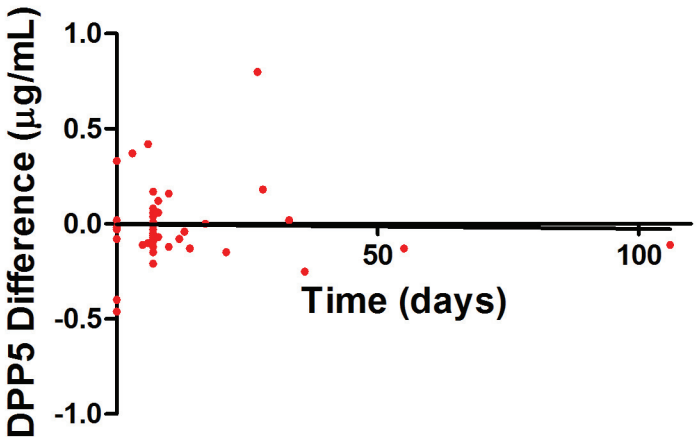

Figure 21- Serial plasma DPP5 levels versus time elapsed. Scatter plot of the difference between the DPP5 levels measured in 1st and 2nd blood samples obtained from 44 cancer patients versus the time elapsed between the 1st and 2nd blood draw. Linear regression line is plotted.

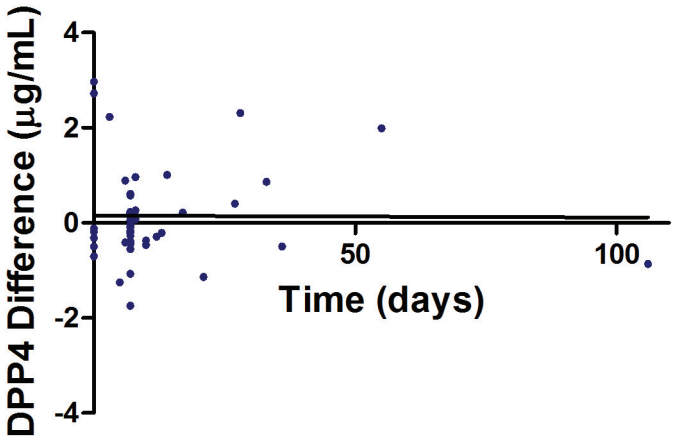

Figure 22- Serial plasma DPP4 levels versus time elapsed. Scatter plot of the difference between the DPP4 levels measured in 1st and 2nd blood samples obtained from 44 cancer patients versus the time elapsed between the 1st and 2nd blood draw. Linear regression line is plotted.

- Association of Plasma DPP5 and DPP4 Levels with Cancer Survival (Specific Aim 3):

Follow-up data were available from 344 cancer patients. Median follow-up duration was 645 (1622) days; 115 (33.2%) of the cases were alive at the last

follow-up visit. There was no significant association between the DPP5 level and the patients' last known survival status (alive versus dead), but DPP4 levels were lower in cancer patients who had died (3.59±2.12 in deceased versus 3.87±1.71 in alive patients, p=0.027). When all cancer patients were considered, patients with longer follow-up duration had higher DPP4 levels (p=0.003). When the analysis was limited to the patients who were alive at the last follow-up, patients with longer duration of follow-up had higher DPP5 levels (p=0.033). There was a similar trend with DPP4, but it fell short of statistical significance (p=0.068).

In Cox regression analysis of all types of cancer 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). When patients were grouped based on their DPP4 level being above or below the median, patients with lower DPP4 levels had shorter survival compared with those with higher DPP4 levels (Log Rank test comparing groups above or below median DPP4 level of 3.43 μg/mL, p=0.021, Figure 23A). In *post hoc* analysis, the difference was most significant when DPP4 level of 3.05 μg/mL (30^{th} percentile) was used as the cut-off point (p=0.001; Figure 23B). Conversely, lower DPP5 was associated with worse survival, only when metastatic cases were excluded (HR 0.637, 95% CI 0.423-0.960 for log-transformed variable, p=0.031, Figure 24).

Subgroup analysis of survival was performed for cancers with adequate sample size as described in the Materials and Methods section. In hematological malignancies, DPP4 and DPP5 levels were similar among leukemia (n=9), lymphoma (n=15) and multiple myeloma (n=4) cases and they were analyzed together. Older age was associated with significantly higher DPP5 levels (p=0.001), but not DPP4 (p=0.786). Head and neck malignancies (Table 2) consisted of 20 cases of oropharyngeal malignancies, 12 laryngeal cancers and 8 thyroid cancer cases. DPP4 and DPP5 levels were similar among different cancer types. Patients with higher DPP5 levels had better survival (Log Rank test comparing groups above or below median DPP5 level of 0.41 μg/mL, p=0.039; HR 0.486, 95% CI 0.213-1.110 for log-transformed variable, p=0.087; Figure 25).

Similarly, head and neck cancer patients with higher DPP4 levels had better survival (Log Rank test comparing groups above or below median DPP4 level of 3.84 µg/mL, p=0.042; HR 0.252, 95% CI 0.079-0.806 for the log-transformed variable, p=0.020; Figure 26A). In *post hoc* analysis, the difference was most significant when DPP4 level of 0.61 µg/mL (20^{th} percentile) was used as the cut-off point (p=0.002; Figure 26B).

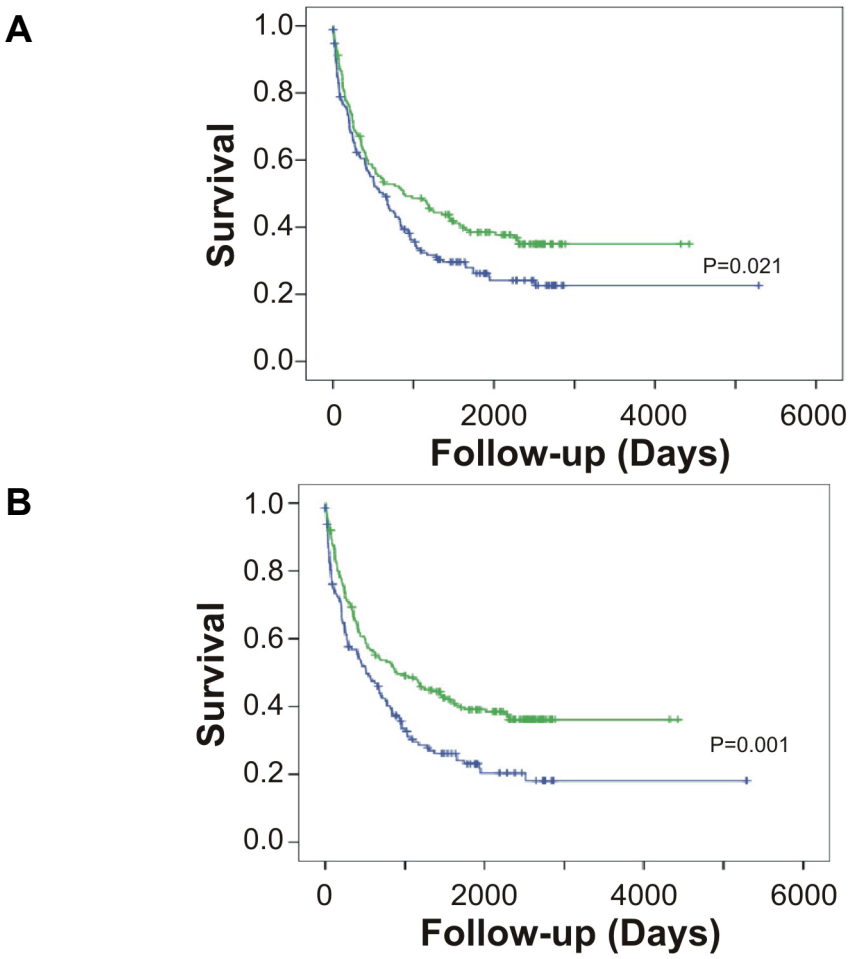

Figure 23- DPP4 and survival in all cancers. Panel A: Survival in cancer patients with DPP4 levels <3.43 µg/mL (median; blue line) versus those with DPP4 levels >3.43 µg/mL (green line). Panel B: Same as panel A, using DPP4 level of 3.05 µg/mL as the cut-off point; Total n=344. Censored cases are denoted by "+" (n=113).

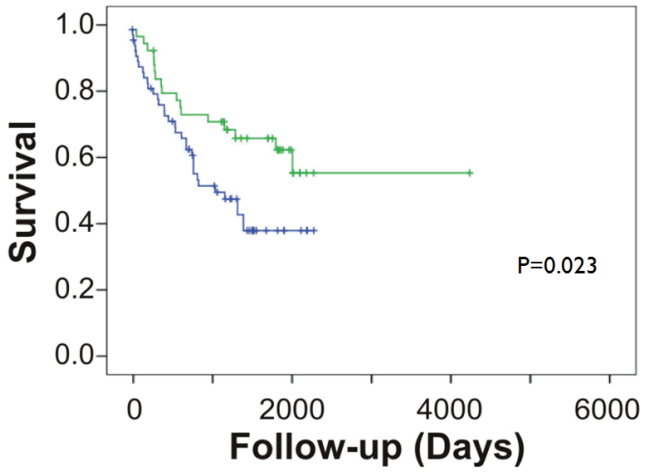

Figure 24- DPP5 and survival in non-metastatic cancers. Survival in non-metastatic cancer patients with DPP5 levels <0.35 µg/mL (median; blue line) versus those with DPP5 levels >0.35 µg/mL (green line); Total n=110. Censored cases are denoted by "+" (n=59).

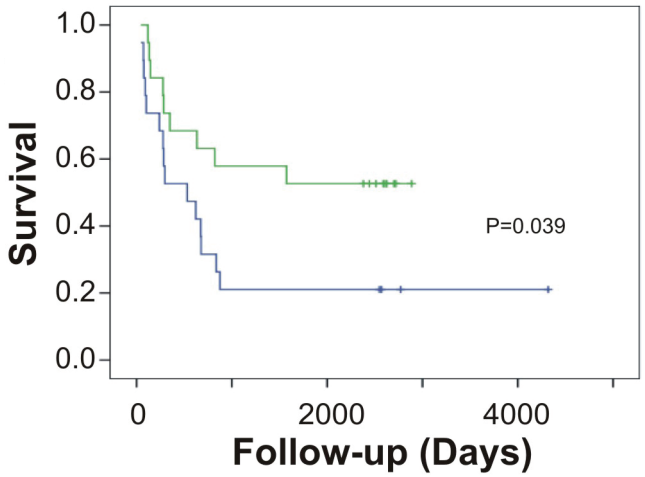

Figure 25- DPP5 and survival in head and neck cancers. Survival in head and neck cancer patients with DPP5 levels <0.41 µg/mL (median; blue line) versus those with DPP5 levels >0.41 µg/mL (green line); Total n=38. Censored cases are denoted by "+" (n=14).

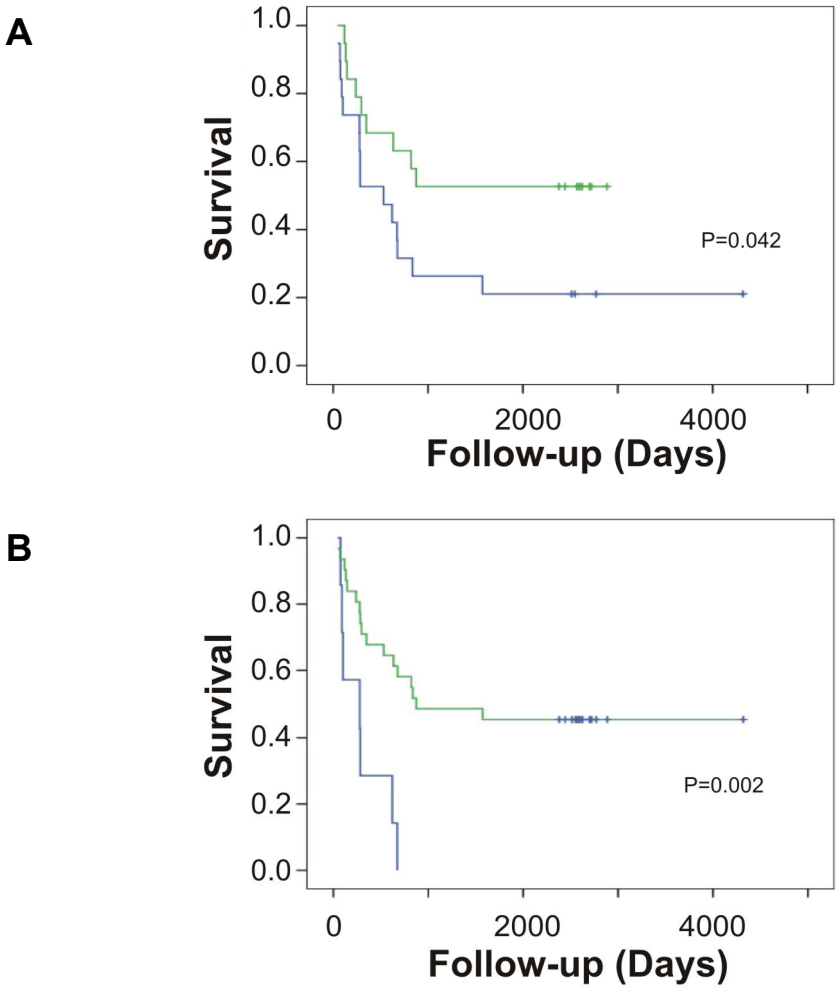

Figure 26- DPP4 and survival in head and neck cancers. Panel A: Survival in head and neck cancer patients with DPP4 levels <3.84 µg/mL (median; blue line) versus those with DPP4 levels >3.84 µg/mL (green line). Panel B: Same as panel A, using DPP4 level of 0.61 µg/mL as the cut-off point; Total n=38. Censored cases are denoted by "+" (n=14).

Lung malignancies consisted of 67 non-small cell and 16 small cell carcinomas. There were no significant differences in DPP4 and DPP5 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 µg/mL) was significantly lower compared with stage I (4.77±0.38 µg/mL, p=0.031) and stage IV (3.95±1.67 µg/mL, p=0.009) cancers. There was a non-significant trend between lower DPP4 levels and shorter survival (HR 0.657, 95% CI 0.401-1.079, p=0.097). In *post hoc* analysis, based on DPP4 cut-off point of 4.47 µg/mL (70th

percentile), patients with higher DPP4 level had better survival (p=0.007; Figure 27).

In gynecologic cancers (Table 2), smaller tumors (T1) had higher DPP4 levels compared with T2-4 tumors (p<0.001). Similarly, stage I tumors had significantly higher DPP4 levels (p<0.001). There was no significant association between DPP4 and DPP5 levels and survival, but there was a trend toward better survival in patients with higher DPP4 levels (HR 0.636, 95% CI 0.385-1.051, p=0.077).

In contrast to other cancers, older age was associated with lower DPP5 levels in patients with colorectal cancer (Table 2), (p=0.016). There was a non-significant trend toward shorter survival in patients with higher DPP5 levels (HR 1.997, 95% CI 0.886-4.500 for the log-transformed variable, p=0.095; Log Rank test comparing groups above or below median DPP5 level of 0.43 µg/mL, p=0.515 [Figure 28A]). In *post hoc* analysis, the difference was most significant when DPP5 level of 0.64 µg/mL (30th percentile) was used as the cut-off point (p=0.003; Figure 28B).

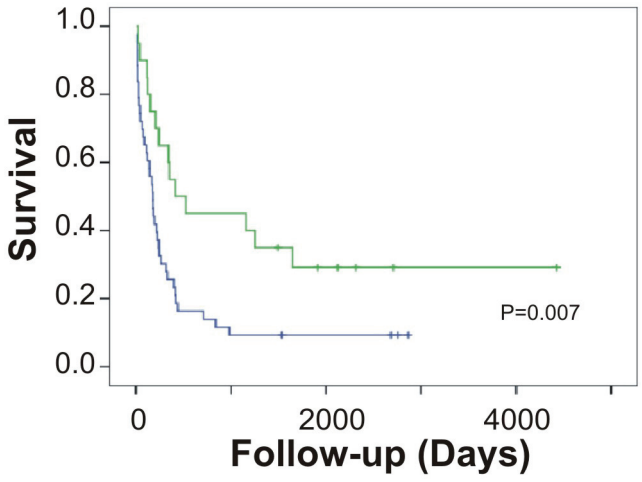

Figure 27- DPP4 and survival in lung cancer. Survival in lung cancer patients with DPP4 levels <4.47 µg/mL (blue line) versus those with DPP4 levels >4.47 µg/mL (green line); Total n=63. Censored cases are denoted by "+" (n=10).

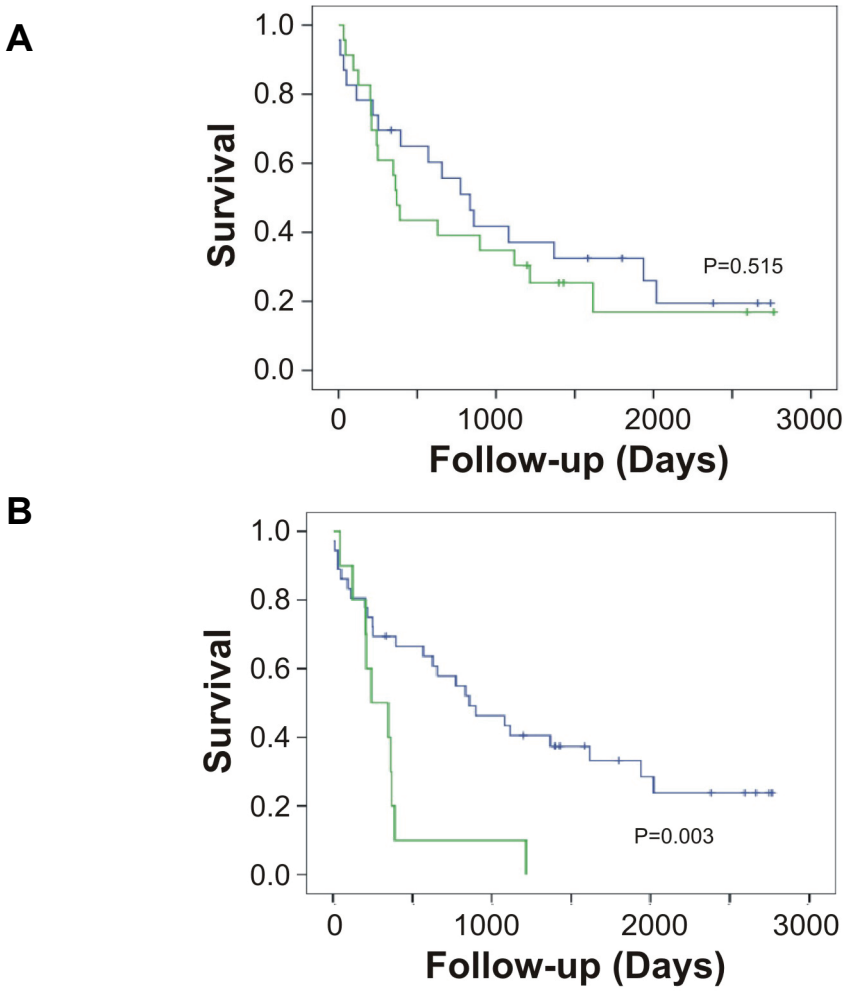

Figure 28- DPP5 and survival in colorectal cancers. Panel A: Survival in colorectal cancer patients with DPP5 levels <0.43 µg/mL (median; blue line) versus those with DPP5 levels >0.43 µg/mL (green line). Panel B: Same as panel A, using DPP5 level of 0.64 µg/mL as the cut-off point; Total n=46. Censored cases are denoted by "+" (n=11).

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 DPP5 (0.68±0.16 µg/mL) levels compared with gastro-esophageal cancers (DPP4: 3.19±1.64 µg/mL, p=0.009; DPP5: 0.39±0.20 µg/mL, p=0.008). Also, DPP5 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/DPP5 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/DPP5 levels and gender in head and neck, lung and colorectal malignancies. No effect of DPP4/DPP5 levels on survival was noted in breast and hematological malignancies. Finally, in gynecologic cancers, there was no association between DPP4/DPP5 levels and patients' age, CA125 levels and circulating tumor cell counts at time of diagnosis (Fan et al., 2009).

- Proteolytic Release of DPP5 from Cells (Specific Aim 4):

LOX (human melanoma) and SB247 (human ovarian carcinoma) cell lines as well as two previously established DPP5-knocked-down variants of LOX cell lines (Chen et al., 2006) were cultured in regular medium and the conditioned medium was assayed for DPP5 and DPP4 presence using ELISA and enzymatic assays. While the LOX-conditioned medium was positive for DPP5, no DPP5 was detected in conditioned medium of knocked-down LOX cells, SB247 cells, or unconditioned medium. No DPP4 was detected in none of the samples (Figure 29). Results were similar when serum-free medium was used.

LOX cell line was used to study the potential proteolytic mechanism of release of DPP5 into culture medium, as an *in vitro* model to explain the source of DPP5 in plasma. In order to rule out the possibility that the detected DPP5 in conditioned medium was associated with membranes (e.g. membrane-bound on vesicles released by LOX cells), conditioned medium was cleared and ultracentrifuged. While there was no visually detectable difference in DPP5 levels before and after ultracentrifugation, DPP5 was detected in the resuspended pellet (Figure 30). Based on ELISA of serial dilutions of resuspended pellet, it was determined that ~80% of the total DPP5 antigen present in LOX conditioned medium was in free form and ~20% was associated with vesicles. The membrane-bound DPP5 was not detectable by ELISA unless released by strong detergents.

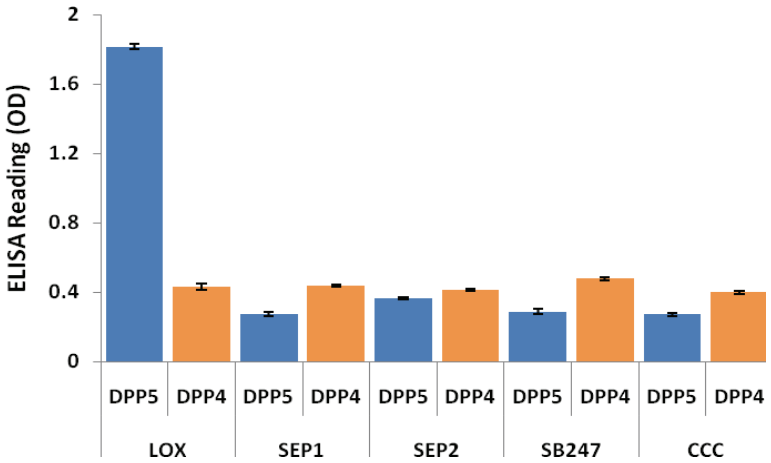

Figure 29- DPP5 in conditioned medium of LOX cells. Various cell lines were cultured in regular medium to near confluent and the conditioned medium was sampled and assayed for DPP4 and DPP5 using ELISA. LOX, regular medium conditioned by LOX cell line; SEP1 and SEP2, regular medium conditioned by DPP5-knocked-down variants of LOX cell line; SB247, regular medium conditioned by SB247 cell line; CCC, unconditioned regular medium. Error bars represent SD of triplicates. Slight rise in the DPP5 level observed in medium conditioned by SEP2 cells is consistent with previous report of incomplete inhibition of DPP5 expression in this cell line (Chen et al., 2006).

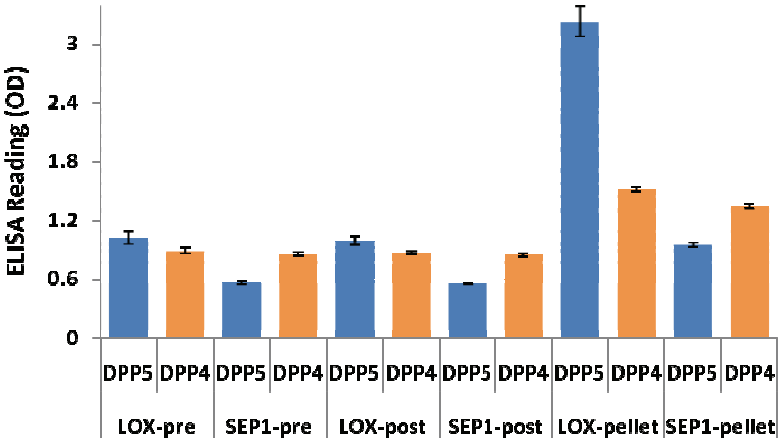

Figure 30- Free versus membrane-bound DPP5. Conditioned medium was ultracentrifuged and assayed for DPP5 and DPP4 levels using ELISA. LOX-pre, LOX cell medium before ultracentrifugation; SEP1-pre, DPP5-knocked-down LOX cell medium before ultracentrifugation; LOX-post, LOX cell medium after ultracentrifugation; SEP1-post, DPP5-knocked-down LOX cell medium after ultracentrifugation; LOX-pellet, resuspended pellet of LOX cell medium after ultracentrifugation; SEP1-pellet, resuspended pellet of DPP5-knocked-down LOX cell medium after ultracentrifugation. Error bars represent SD of triplicates.

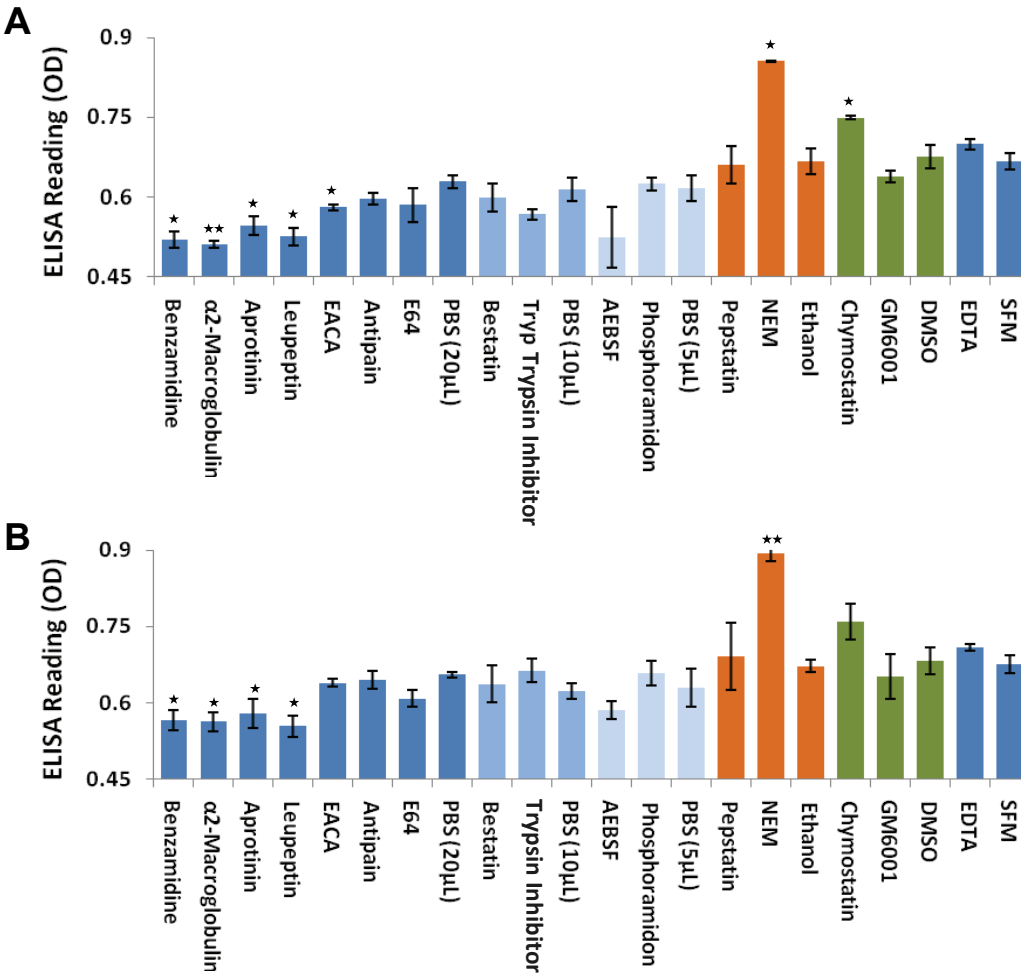

Figure 31- Effect of protease inhibitors on free DPP5 level. Conditioned medium of LOX cells in presence of various protease inhibitors or controls, sampled at 3 (panel A) and 6 hours (panel B) was assayed for DPP5 level using ELISA. The bars are clustered together in the same color based on the volume and type of solvent used to add the inhibitor, and the last bar in each color cluster represents the solvent with no inhibitor (control; 20, 10, and 5 µL PBS, Ethanol, DMSO, and SFM [serum-free DMEM]). Error bars represent SD of triplicates. *) $p<0.01$ compared with the respective control; **) $p<0.001$ compared with the respective control.

Following confirmation that LOX cells release soluble DPP5 into their culture medium, LOX cells were cultured, resuspended using trypsin or PBS, washed and replated in presence of various protease inhibitors (Table 1). Conditioned medium was sampled and assayed for DPP5 concentration using ELISA at 3 hours (Figure 31A) and 6 hours (Figure 31B). Results were comparable using trypsin or

PBS to resuspend the cells. Cell viability at 3 and 6 hours was >95% in all wells. The results of 4 independent experiments are summarized in Table 5. The data show that inhibitors of serine proteases are most frequently associated with reduced DPP5 in the conditioned medium. Conversely, NEM (a cysteine protease) is associated with increased DPP5. Finally, inhibitors of metalloproteases did not affect the ELISA-detected level of DPP5 in LOX conditioned medium.

Table 5- Summary of effect of various protease inhibitors on DPP5 level. The data from 4 independent experiments are combined. "↓" indicates reduction and "↑" indicates increase of DPP5 level in conditioned medium compared with control. Based on t-tests results, for each $p<0.01$ one (↓ or ↑) and for each $p<0.001$ two (↓↓ or ↑↑) scores were given. "↔" indicates no significant ($p<0.01$) difference from the control in any experiments.

Inhibitor	Effect on [DPP5]	Inhibitory Action of the Inhibitor
Benzamidine	↓↓↓↓	Serine proteases, trypsin
α2Macroglobulin	↓↓↓↓↓	Serine/cysteine/aspartic/metalloproteases
Aprotinin	↓↓	Serine protease
Leupeptin	↓↓↓↓	Serine/cysteine proteases
EACA	↓	Chymotrypsin, FVIIa, lysine carboxypeptidase, plasmin, plasminogen activator
Antipain	↓	Serine/cysteine proteases
E64	↓	Cysteine proteases
Bestatin	↓	Leucine aminopeptidase/triamino peptidase
Trypsin Inhibitor	↓	Trypsin/chymotrypsin/plasmin
AEBSF	↓↓	Serine proteases
Phosphoramidon	↔	Bacterial metalloproteases
Pepstatin	↓	Aspartyl proteases
NEM	↑↑↑	Cysteine proteases
Chymostatin	↑	Chymotrypsin-like serine proteases, chymases, lysosomal cysteine proteases
GM6001	↔	MMPs
EDTA	↔	Metalloproteases

DISCUSSION:

There has been considerable interest in the role of DPP4 in various pathophysiologic processes and as a novel therapeutic target for diabetes, brain tumors and infectious diseases (Yazbeck et al., 2009; Busek et al., 2008; Havre et al., 2008; Yu et al., 2010). The other major member of the DPP family, DPP5, is of interest because of the demonstration of its high expression in solid tumor tissues (O'Brien and O'Connor, 2008). DPP5 has also been reported to be a promising diagnostic and therapeutic target in malignancies (Aoyama and Chen, 1990; Chen et al., 2006; Christiansen et al., 2007; Kennedy et al., 2009; Narra et al., 2007; Ostermann et al., 2008; Scott et al., 2003). Although DPP5 has been isolated from plasma, the clinical relevance of soluble DPP5 in blood in cancer patients has not yet been addressed in published reports (Lee et al., 2006; Narra et al., 2007).

In this study, a novel ELISA for measurement of DPP5 in plasma was developed and validated to be sensitive and specific, and yield reproducible results. The ELISA measurements were proportional to the DPP activity of DPP4 and DPP5 in plasma samples, despite the fact that the plasma samples had undergone at least two freeze-thaw cycles and this could affect the enzymatic activity. This observation suggests that the antigenic level of DPP4 and DPP5 in plasma as measured by ELISA (at least in the limited number of samples studied) is proportional to the level of enzymatic activity of DPP4 and DPP5, which further supports the validity of the measurements.

A large series of plasma samples from healthy donors and cancer patients was assayed for DPP5 and DPP4. This study demonstrated new findings with regards to plasma DPP5 and confirmed previously-reported correlations regarding DPP4. Plasma DPP4 and DPP5 levels were higher in healthy male than female subjects. Using DPP4 and DPP5 levels was demonstrated to be an accurate test to discriminate between the healthy individuals and cancer patients, particularly in

non-metastatic cases. Previous reports support DPP4 as a prognostic factor in some types of cancer, with patients with higher levels of DPP4 experiencing longer survival. This was confirmed when all cancer cases were combined, as well as in lung and head and neck cancer subgroup analyses in this study. A similar trend was observed with DPP5 in head and neck cancers. The notable exception was the case of colorectal carcinomas in which higher DPP5 levels were associated with poor prognosis. Previous studies demonstrated that higher local expression of DPP5 in colorectal tumors is associated with more lymph nodal involvement, more metastasis, and poor survival (Henry et al., 2007; Iwasa et al., 2005). However, the present study did not detect a statistically significant association between plasma DPP5 level and lymph node involvement or metastasis. Conversely, another study has reported higher tumor stromal DPP5 staining in earlier stage colorectal tumors, as well as decreased survival in high-DPP5 staining metastatic colorectal tumors, which is in line with present study's findings (Henry et al., 2007).

The average DPP4 level in plasma of healthy adults has been reported to be approximately 7 μg/mL (Gorrell et al., 2001). Using the DPP4 ELISA developed in this study, the mean DPP4 level in plasma samples from healthy adults was 5.69±1.47 μg/mL, which is remarkably close to the previously reported level. Measured plasma DPP5 levels were nearly 9-fold lower (0.66±0.19 μg/mL in healthy subjects) compared with DPP4 levels in the same individuals. Reports indicate that only around 90% of the DPP activity in human plasma can be attributed to plasma DPP4, and the source of the remaining 10% of activity has been a mystery (Cordero et al., 2009). Based on this study, plasma DPP5 can be suggested as a likely candidate to explain the remaining DPP activity in blood.

Interestingly, plasma DPP4 and DPP5 levels in this study closely correlated with each other, with each protein level capable of predicting some 38% of variability of the other protein level. DPP4 and DPP5 are known to form hetero-oligomeric complexes on the cell membrane (Chen et al., 2006; Chen, 2003; Ghersi et al., 2002; Ghersi et al., 2003; Ghersi et al., 2006). It is possible

that similar complexes exist in blood circulation, which could explain the correlation of DPP4 and DPP5 levels in plasma, although preliminary experiments at our lab failed to co-isolate detectable levels of DPP4, when DPP5 was captured from plasma using mAb-coated beads (Western blotting) or vice versa using enzymatic assays in mAb-coated microplates to capture DPP4 (unpublished data).

We demonstrated that plasma DPP5 levels in 561 cancer patients were significantly lower compared with healthy subjects (0.46±0.30 versus 0.66±0.19μg/mL, respectively). The finding was confirmed to be independent of subjects' age or gender. This was an unexpected finding in light of extensive evidence showing high local concentration of DPP5 in various solid tumor tissues (Fluge et al., 2006; Mori et al., 2004; Goscinski et al., 2008; Cohen et al., 2008; Dolznig et al., 2005; Garin-Chesa et al., 1990; Iwasa et al., 2005; Kennedy et al., 2009; Okada et al., 2003; Zhang et al., 2007). Increased local expression of DPP5 has been reported in lung, breast, colorectal, ovarian, head and neck, bladder, skin and pancreatic tumors as well as some sarcomas. Nonetheless, as can be seen from Table 2, plasma from patients with most cancer types had DPP5 levels lower than healthy subjects, with some (gynecologic, hematologic, lung and head and neck tumors) displaying particularly low plasma DPP5 levels. It remains to be determined if the lower level of plasma DPP5 seen in cancer patients is a nonspecific change in line with reduction of some other plasma proteins such as albumin and other negative acute-phase proteins seen in inflammatory processes and malignancies (e.g. transferrin and inter-alpha-trypsin inhibitors (Ritchie et al., 1999; Daveau et al., 1993)), or a more specific phenomenon. Possible (and speculative) explanations for the observed lower level in cancer patients include active uptake of DPP5 from plasma by tumor cells, removal by the reticuloendothelial system (alone, or in combination with other molecules such as auto-antibodies), binding to other plasma proteins that mask antibody-binding epitopes (e.g. auto-antibodies), protein degradation/truncation, or accumulation in thrombi (see below). ELISAs to quantify putative DPP5 autoantibodies in plasma samples have been developed at our lab for future investigation.

Of note, the method used in this study (sandwich ELISA) relies on recognition and binding of certain epitopes on DPP5 by 2 different mAbs, and various phenomena such as masking of either epitope or truncation of the protein and dissociation of the binding sites of the 2 mAbs can interfere with the assay readings (Figure 32). Following spiking rDPP4 in plasma samples, limited recovery of rDPP4 was observed by the assay (Figure 8), which may be due to trapping of rDPP4 by molecules present in plasma (e.g. α2 macroglobulin) and masking of the epitopes that bind the ELISA monoclonal antibodies. Nonetheless, a similar limited recovery was not observed following spiking of plasma with rDPP5.

Western blotting data using E97 mAb in this study indicate that most of DPP5 in human plasma is truncated as indicated by the 25-KDa size of the predominant band under denaturing conditions (Figure 2). However, the DPP5 mAbs used to develop ELISA in this study (D8 and D28) are known to recognize the larger (active) 97-KDa monomer of DPP5, not the smaller (inactive) 25-KDa fragment (Chen et al., 2006). Nonetheless, given the lack of gelatinase and DPP activity in 25-KDa fragments detected by E97, and the inability of E97 to recognize native active DPP5 in dimeric form (the expected active form present in plasma) which prevented its application in developing ELISA, use of D8 and D28 mAbs in developing the ELISA in this study was justified.

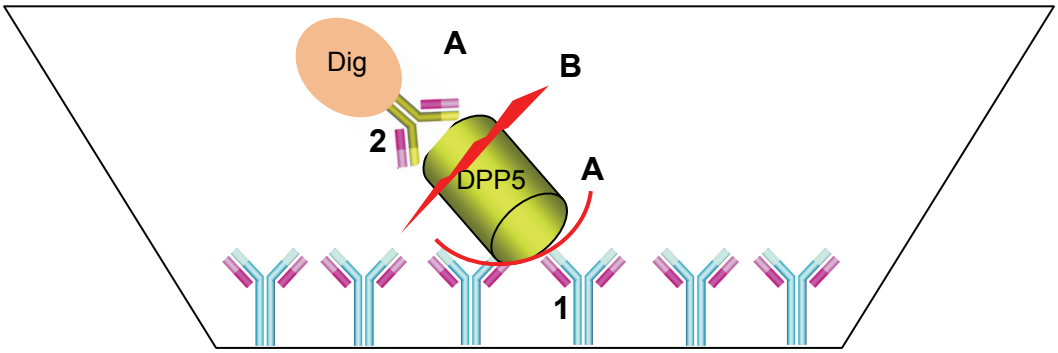

Figure 32- Potential Interferences with DPP5 ELISA. Sandwich ELISA relies on binding of the target protein to immobilized D28 mAbs (1) on the plate, and binding of digoxigenin-conjugated D8 mAb (2) to DPP5. Various factors can interfere with this process and potentially affect the results. A) Other molecules masking either mAb-binding epitopes on DPP5; B) Truncation of DPP5, resulting in physical separation of mAb-binding sites. Dig, digoxigenin.

Decreased plasma DPP4 levels (as well as discrepancies between DPP4 antigenicity measured by ELISA versus DPP4 activity measured by DPP enzymatic assays) have been observed in some cancers, and differences in glycosylation patterns affecting immunorecognition of DPP4 or interaction with other proteins have been suggested as possible reasons (Cordero et al., 2009). Similar possibilities exists for DPP5, although some data suggest glypican 3 (a proteoglycan on the cell surface) to be the only natural inhibitor of DPP5, undermining the possibility of plasma molecules such as α2 macroglobulin or α1 anti-trypsin being responsible for removal of DPP5 from plasma and affecting its plasma level (Davoodi et al., 2007). Additionally, given the role of DPP4 in activating immune response, and its increased plasma level in inflammatory conditions, it has been suggested that some tumors may actively inhibit DPP4 expression from T cells by expressing immunosuppressive proteins such as TGF-β_1, thus inducing tolerance toward themselves (Cordero et al., 2009). There is the possibility that such immunosuppressive signals may (inadvertently) affect DPP5 expression as well, given the similarity of the two proteases. Interestingly, it has recently been reported that TGF-β may induce expression of DPP5 in a highly metastatic human ovarian cancer cell line (Chen et al., 2009), although more evidence is needed to substantiate this finding.

While the source of plasma DPP5 is unknown, its presence in plasma of healthy subjects indicates physiologic source(s). On the other hand, membrane-bound DPP5 can be proteolytically cleaved and released from the tumor cell surface as demonstrated in this study (Figure 29), and as previously reported (Chen et al., 2006). This phenomenon would suggest that similar to some other proteases, plasma DPP5 level should increase in malignancy as the number of tumor-associated DPP5-expressing cells increase (Zucker et al., 2004). One speculation to explain our data is that as the tumor burden increases, the amount of plasma DPP5 derived from physiologic sources declines, while the tumor cell-derived DPP5 increases particularly in advanced disease (stage IV) (Figure 33). Consistent with this hypothesis, this study shows that serine protease inhibitors are capable of reducing the level of free DPP5 in media conditioned by metastatic

melanoma cells, suggestive of increasing proteolytic shedding of DPP5 from stage IV tumors.

As displayed in Figure 18, mean plasma DPP5 levels declined as cancer progress from stage I to stage III, and then increased in stage IV. Interestingly, a near-significant trend was observed toward higher plasma DPP5 levels in metastatic cases. More studies are needed to determine if there is a clinical application for this phenomenon (e.g. as a tool for monitoring disease progress and early detection of metastasis). Similar phenomenon was observed for DPP4 (Figure 17), and this observation is consistent with previous reports of increased plasma DPP4 levels in stage IV cancers (Cordero et al., 2009).

It is generally believed that bulk of plasma DPP4 originates from hepatobiliary system followed by immune system, through proteolytic release and shedding of the membrane-bound form of DPP4 into blood circulation (Cordero et al., 2009). This study demonstrated that serine protease inhibitors are capable of reducing the level of free DPP5 in LOX conditioned medium, suggestive of a similar proteolytic shedding mechanism for release of DPP5 into plasma, mediated by serine proteases. Given that DPP5 is a serine protease, it remains to be determined if DPP5 can act on itself to release it from the cell surface. It has been previously demonstrated that proteolytic truncation of DPP5 in conditioned medium is mediated by (yet to be identified) metalloproteases (Chen et al., 2006). However, in the model utilized in the present study, metalloprotease inhibitors did not affect the detected level of DPP5 in conditioned medium (Table 5), suggesting that metalloproteases are not involved in shedding of DPP5 from cells, and metalloprotease-truncated forms of DPP5 are still detectable by the ELISA.

Given the similarities between DPP4 and DPP5, the sources of plasma DPP4 (hepatobiliary and immune system) should be considered when searching for physiologic sources of plasma DPP5. In this study, there was only 1 patient with liver cancer. Although DPP4 and DPP5 levels of this patient were both above the normal average (8.82 μg/mL and 0.73 μg/mL, respectively), no conclusion can be drawn based on a single case. Nonetheless, relatively high plasma DPP5 levels were also observed in pancreatic cancers, which may be a

clue to the liver or pancreas being the physiologic source of plasma DPP5, given the proximity of pancreas to liver and its common involvement in pancreatic cancers, as previously discussed. Conversely, mean DPP4 and DPP5 levels in leukemia and lymphomas in this study were lower compared with healthy subjects (Table 2).

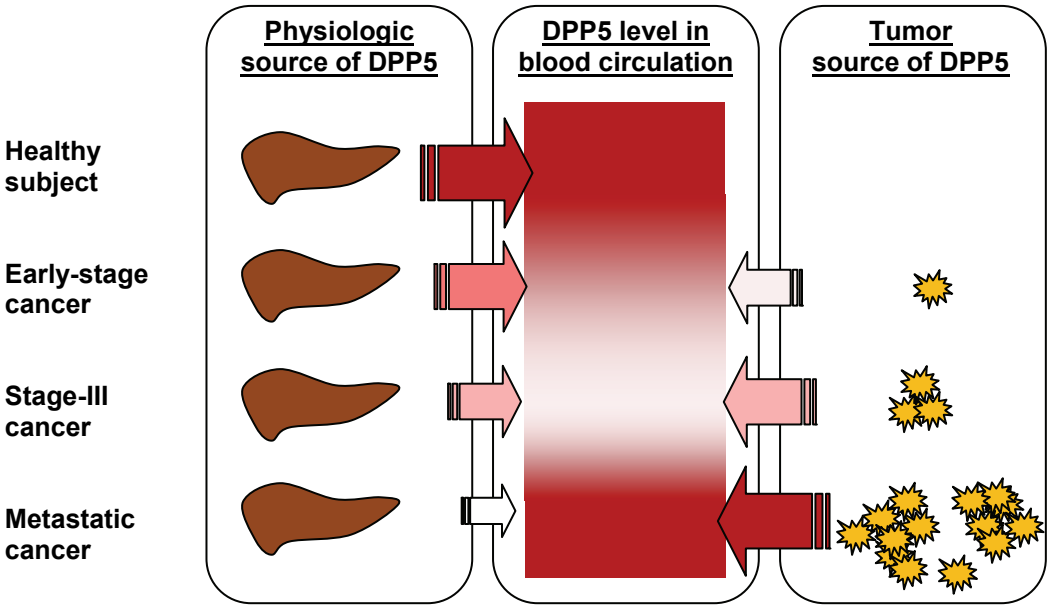

Figure 33- "Dual source" theory of plasma DPP5 in cancer. As cancer progresses, the DPP5 derived from the physiologic source declines while the DPP5 derived from tumor increases.

Contrary to most tumor types, breast and prostate cancer patients did not exhibit lower-than-normal DPP5 levels. The fact that the mean age of breast and prostate cancer patients was similar to other types of cancer displaying low levels of DPP5 (Table 2) tends to negate an important impact of age on DPP5 measurements. In breast cancer patients, the predominance of lower stage cancers (median stage of II in breast cancer cases as opposed to median stage IV for other types of cancer) may be related to the near-normal observed plasma levels of DPP5. This explanation does not apply to prostate cancers, which were predominantly stage IV. The hormonal dependence of breast and prostate tumors is another factor for future consideration. Interestingly, a previous study of DPP5

expression levels in tumors (using RT-PCR) reported that breast, uterus, and cervix had the highest levels of expression among the normal tissues (Dolznig et al., 2005). Of the malignant tissues studied, breast cancers (ductal and lobular carcinomas) had significantly higher DPP5 expression compared with normal breast tissue, while the expression levels of malignant uterus/cervix tissues did not differ from normal tissues.

Previous studies have described an antifibrinolytic role for DPP5 in the circulation, possibly favoring the production/maintenance of thrombosis (Lee et al., 2006). A potential relationship between plasma DPP5 level and the coagulation status of cancer patients, who are prone to thromboembolic events resulting in considerable morbidity and mortality, is an appropriate subject for further research. Notably, it is yet to be determined if higher plasma levels of DPP5 (e.g. in stage IV cancers) can be used to predict the risk of thromboembolic events in these patients, and if so, be used in deciding on thromboembolic prophylaxis.

The results of this study should be interpreted with consideration of its limitations. Although the ELISA methodology developed for this study has been extensively tested and validated, the presence of other molecules interacting with DPP5 and DPP4 in blood, e.g. autoantibodies and other binding macromolecules, can potentially interfere with ELISA results. Each patient in this study usually provided only one (or two) blood samples and having access to more blood samples during the follow-up period could have allowed longitudinal analysis to study the possible change of DPP4 and DPP5 levels through the course of therapy and disease. Unavailable and missing demographic/clinical data for a substantial number of the patients is another limiting factor.

Despite these limitations, this study is the first to address the levels of DPP5 and DPP4 in plasma of healthy subjects and cancer patients and their association with tumor type, stage, and survival. It is hoped that this study will serve as the basis for further in-depth analyses of the clinical significance of plasma DPP5 and DPP4 in various types of cancers as a potential diagnostic and prognostic factor, as well as the use of circulating DPP5 and DPP4 measurements in monitoring (and adjusting) treatments employing DPP5 and DPP4 inhibitors.

CONCLUSION

This study demonstrates that plasma DPP5 and DPP4 levels are reproducible parameters that correlate with disease status, with lower levels noted in cancer patients as compared with healthy subjects, as well as higher stage non-metastatic cancers compared with early-stage cancers. Higher DPP5 levels are generally associated with better cancer prognosis, although this relationship may be inversed in stage IV cancers. An *in vitro* model supports proteolytic shedding of membrane-bound DPP5 by serine proteases as the potential source of plasma DPP5. All together, these findings support a prognostic role for plasma DPP5 and DPP4 levels in cancers and their plasma levels should be considered in designing clinical investigations focusing on DPP5 as a therapeutic target to hinder cancer progression. An explanation for low levels of plasma DPP5 in cancer despite previously-reported high level of local expression, and physiologic source(s) of DPP5 remain to be determined.

BIBLIOGRAPHY

Aoyama,A. and Chen,W.T. (1990). A 170-kDa membrane-bound protease is associated with the expression of invasiveness by human malignant melanoma cells. Proc. Natl. Acad. Sci. U. S. A *87*, 8296-8300.

Bae,S., Park,C.W., Son,H.K., Ju,H.K., Paik,D., Jeon,C.J., Koh,G.Y., Kim,J., and Kim,H. (2008). Fibroblast activation protein alpha identifies mesenchymal stromal cells from human bone marrow. Br. J Haematol. *142*, 827-830.

Bauer,S., Jendro,M.C., Wadle,A., Kleber,S., Stenner,F., Dinser,R., Reich,A., Faccin,E., Godde,S., Dinges,H., Muller-Ladner,U., and Renner,C. (2006). Fibroblast activation protein is expressed by rheumatoid myofibroblast-like synoviocytes. Arthritis Res. Ther. *8*, R171.

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

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

Chen,H., Yang,W.W., Wen,Q.T., Xu,L., and Chen,M. (2009). TGF-beta induces fibroblast activation protein expression; fibroblast activation protein expression increases the proliferation, adhesion, and migration of HO-8910PM [corrected]. Exp. Mol. Pathol. *87*, 189-194.

Chen,W.T. (2003). DPPIV and seprase in cancer invasion and angiogenesis. Adv. Exp. Med. Biol. *524*, 197-203.

Chen,W.T. and Kelly,T. (2003). Seprase complexes in cellular invasiveness. Cancer Metastasis Rev. *22*, 259-269.

Chen,W.T., Kelly,T., and Ghersi,G. (2003). DPPIV, seprase, and related serine peptidases in multiple cellular functions. Curr. Top. Dev. Biol. *54*, 207-232.

Chen,W.T., Olden,K., Bernard,B.A., and Chu,F.F. (1984). Expression of transformation-associated protease(s) that degrade fibronectin at cell contact sites. J. Cell Biol. *98*, 1546-1555.

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

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

Collins,P.J., McMahon,G., O'Brien,P., and O'Connor,B. (2004). Purification, identification and characterisation of seprase from bovine serum. Int. J. Biochem. Cell Biol. *36*, 2320-2333.

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

Daveau,M., Rouet,P., Scotte,M., Faye,L., Hiron,M., Lebreton,J.P., and Salier,J.P. (1993). Human inter-alpha-inhibitor family in inflammation: simultaneous synthesis of positive and negative acute-phase proteins. Biochem. J. *292 (Pt 2)*, 485-492.

Davoodi,J., Kelly,J., Gendron,N.H., and MacKenzie,A.E. (2007). The Simpson-Golabi-Behmel syndrome causative glypican-3, binds to and inhibits the dipeptidyl peptidase activity of CD26. Proteomics. *7*, 2300-2310.

Dohi,O., Ohtani,H., Hatori,M., Sato,E., Hosaka,M., Nagura,H., Itoi,E., and Kokubun,S. (2009). Histogenesis-specific expression of fibroblast activation protein and dipeptidylpeptidase-IV in human bone and soft tissue tumours. Histopathology *55*, 432-440.

Dolznig,H., Schweifer,N., Puri,C., Kraut,N., Rettig,W.J., Kerjaschki,D., and Garin-Chesa,P. (2005). Characterization of cancer stroma markers: in silico analysis of an mRNA expression database for fibroblast activation protein and endosialin. Cancer Immun. *5*, 10.

Fan,T., Zhao,Q., Chen,J.J., Chen,W.T., and Pearl,M.L. (2009). Clinical significance of circulating tumor cells detected by an invasion assay in peripheral blood of patients with ovarian cancer. Gynecol. Oncol. *112*, 185-191.

Fluge,O., Bruland,O., Akslen,L.A., Lillehaug,J.R., and Varhaug,J.E. (2006). Gene expression in poorly differentiated papillary thyroid carcinomas. Thyroid *16*, 161-175.

Garin-Chesa,P., Old,L.J., and Rettig,W.J. (1990). Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. Proc. Natl. Acad. Sci. U. S. A *87*, 7235-7239.

Ge,Y., Zhan,F., Barlogie,B., Epstein,J., Shaughnessy,J., Jr., and Yaccoby,S. (2006). Fibroblast activation protein (FAP) is upregulated in myelomatous bone and supports myeloma cell survival. Br. J. Haematol. *133*, 83-92.

Ghersi,G., Dong,H., Goldstein,L.A., Yeh,Y., Hakkinen,L., Larjava,H.S., and Chen,W.T. (2002). Regulation of fibroblast migration on collagenous matrix by a cell surface peptidase complex. J. Biol. Chem. *277*, 29231-29241.

Ghersi,G., Dong,H., Goldstein,L.A., Yeh,Y., Hakkinen,L., Larjava,H.S., and Chen,W.T. (2003). Seprase-dPPIV association and prolyl peptidase and gelatinase activities of the protease complex. Adv. Exp. Med. Biol. *524*, 87-94.

Ghersi,G., Zhao,Q., Salamone,M., Yeh,Y., Zucker,S., and Chen,W.T. (2006). The protease complex consisting of dipeptidyl peptidase IV and seprase plays a role in the migration and invasion of human endothelial cells in collagenous matrices. Cancer Res. *66*, 4652-4661.

Goldstein,L.A. and Chen,W.T. (2000). Identification of an alternatively spliced seprase mRNA that encodes a novel intracellular isoform. J. Biol. Chem. *275*, 2554-2559.

Goldstein,L.A., Ghersi,G., Pineiro-Sanchez,M.L., Salamone,M., Yeh,Y., Flessate,D., and Chen,W.T. (1997). Molecular cloning of seprase: a serine integral membrane protease from human melanoma. Biochim. Biophys. Acta *1361*, 11-19.

Gorrell,M.D., Gysbers,V., and McCaughan,G.W. (2001). CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes. Scand. J. Immunol. *54*, 249-264.

Goscinski,M.A., Suo,Z.H., Nesland,J.M., Chen,W.T., Zakrzewska,M., Wang,J., Zhang,S., Florenes,V.A., and Giercksky,K.E. (2008). Seprase, dipeptidyl peptidase IV and urokinase-type plasminogen activator expression in dysplasia and invasive squamous cell carcinoma of the esophagus. A study of 229 cases from Anyang Tumor Hospital, Henan Province, China. Oncology *75*, 49-59.

Havre,P.A., Abe,M., Urasaki,Y., Ohnuma,K., Morimoto,C., and Dang,N.H. (2008). The role of CD26/dipeptidyl peptidase IV in cancer. Front Biosci. *13*, 1634-1645.

Henry,L.R., Lee,H.O., Lee,J.S., Klein-Szanto,A., Watts,P., Ross,E.A., Chen,W.T., and Cheng,J.D. (2007). Clinical implications of fibroblast activation protein in patients with colon cancer. Clin. Cancer Res. *13*, 1736-1741.

Huang,Y., Wang,S., and Kelly,T. (2004). Seprase promotes rapid tumor growth and increased microvessel density in a mouse model of human breast cancer. Cancer Res. *64*, 2712-2716.

Iwaki-Egawa,S., Watanabe,Y., Kikuya,Y., and Fujimoto,Y. (1998). Dipeptidyl peptidase IV from human serum: purification, characterization, and N-terminal amino acid sequence. J. Biochem. *124*, 428-433.

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

Kelly,T. (2005). Fibroblast activation protein-alpha and dipeptidyl peptidase IV (CD26): cell-surface proteases that activate cell signaling and are potential targets for cancer therapy. Drug Resist. Updat. *8*, 51-58.

Kennedy,A., Dong,H., Chen,D., and Chen,W.T. (2009). Elevation of seprase expression and promotion of an invasive phenotype by collagenous matrices in ovarian tumor cells. Int. J. Cancer *124*, 27-35.

Lebeau,A.M., Brennen,W.N., Aggarwal,S., and Denmeade,S.R. (2009). Targeting the cancer stroma with a fibroblast activation protein-activated promelittin protoxin. Mol. Cancer Ther. *8*, 1378-1386.

Lee,K.N., Jackson,K.W., Christiansen,V.J., Lee,C.S., Chun,J.G., and McKee,P.A. (2006). Antiplasmin-cleaving enzyme is a soluble form of fibroblast activation protein. Blood *107*, 1397-1404.

Messerschmidt,S.K., Musyanovych,A., Altvater,M., Scheurich,P., Pfizenmaier,K., Landfester,K., and Kontermann,R.E. (2009). Targeted lipid-coated nanoparticles: delivery of tumor necrosis factor-functionalized particles to tumor cells. J. Control Release *137*, 69-77.

Milner,J.M., Kevorkian,L., Young,D.A., Jones,D., Wait,R., Donell,S.T., Barksby,E., Patterson,A.M., Middleton,J., Cravatt,B.F., Clark,I.M., Rowan,A.D., and Cawston,T.E. (2006). Fibroblast activation protein alpha is expressed by chondrocytes following a pro-inflammatory stimulus and is elevated in osteoarthritis. Arthritis Res. Ther. *8*, R23.

Monsky,W.L., Lin,C.Y., Aoyama,A., Kelly,T., Akiyama,S.K., Mueller,S.C., and Chen,W.T. (1994). A potential marker protease of invasiveness, seprase, is localized on invadopodia of human malignant melanoma cells. Cancer Res. *54*, 5702-5710.

Mori,Y., Kono,K., Matsumoto,Y., Fujii,H., Yamane,T., Mitsumata,M., and Chen,W.-T. (2004). The expression of a type II transmembrane serine protease (Seprase) in human gastric carcinoma. Oncology *67*, 411-419.

Narra,K., Mullins,S.R., Lee,H.O., Strzemkowski-Brun,B., Magalong,K., Christiansen,V.J., McKee,P.A., Egleston,B., Cohen,S.J., Weiner,L.M., Meropol,N.J., and Cheng,J.D. (2007). Phase II trial of single agent Val-boroPro

(Talabostat) inhibiting Fibroblast Activation Protein in patients with metastatic colorectal cancer. Cancer Biol. Ther. *6*, 1691-1699.

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

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

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

Park,J.E., Lenter,M.C., Zimmermann,R.N., Garin-Chesa,P., Old,L.J., and Rettig,W.J. (1999). Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. J. Biol. Chem. *274*, 36505-36512.

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

Pure,E. (2009). The road to integrative cancer therapies: emergence of a tumor-associated fibroblast protease as a potential therapeutic target in cancer. Expert. Opin. Ther. Targets. *13*, 967-973.

Ritchie,R.F., Palomaki,G.E., Neveux,L.M., Navolotskaia,O., Ledue,T.B., and Craig,W.Y. (1999). 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*, 273-279.

Saadi,A., Shannon,N.B., Lao-Sirieix,P., O'Donovan,M., Walker,E., Clemons,N.J., Hardwick,J.S., Zhang,C., Das,M., Save,V., Novelli,M., Balkwill,F., and Fitzgerald,R.C. (2010). Stromal genes discriminate preinvasive from invasive disease, predict outcome, and highlight inflammatory pathways in digestive cancers. Proc. Natl. Acad. Sci. U. S. A *107*, 2177-2182.

Santos,A.M., Jung,J., Aziz,N., Kissil,J.L., and Pure,E. (2009). Targeting fibroblast activation protein inhibits tumor stromagenesis and growth in mice. J. Clin. Invest *119*, 3613-3625.

Scanlan,M.J., Raj,B.K., Calvo,B., Garin-Chesa,P., Sanz-Moncasi,M.P., Healey,J.H., Old,L.J., and Rettig,W.J. (1994). Molecular cloning of fibroblast activation protein alpha, a member of the serine protease family selectively

expressed in stromal fibroblasts of epithelial cancers. Proc. Natl. Acad. Sci. U. S. A *91*, 5657-5661.

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

Taverna,S., Ghersi,G., Ginestra,A., Rigogliuso,S., Pecorella,S., Alaimo,G., Saladino,F., Dolo,V., Dell'Era,P., Pavan,A., Pizzolanti,G., Mignatti,P., Presta,M., and Vittorelli,M.L. (2003). Shedding of membrane vesicles mediates fibroblast growth factor-2 release from cells. J. Biol. Chem. *278*, 51911-51919.

Wang,X.M., Yao,T.W., Nadvi,N.A., Osborne,B., McCaughan,G.W., and Gorrell,M.D. (2008). Fibroblast activation protein and chronic liver disease. Front Biosci. *13*, 3168-3180.

Welt,S., Divgi,C.R., Scott,A.M., Garin-Chesa,P., Finn,R.D., Graham,M., Carswell,E.A., Cohen,A., Larson,S.M., and Old,L.J. (1994). 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*, 1193-1203.

Wolf,B.B., Quan,C., Tran,T., Wiesmann,C., and Sutherlin,D. (2008). On the edge of validation - cancer protease fibroblast activation protein. Mini. Rev. Med. Chem. *8*, 719-727.

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

Yu,D.M., Yao,T.W., Chowdhury,S., Nadvi,N.A., Osborne,B., Church,W.B., McCaughan,G.W., and Gorrell,M.D. (2010). The dipeptidyl peptidase IV family in cancer and cell biology. FEBS J *277*, 1126-1144.

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

Zhi,K., Shen,X., Zhang,H., and Bi,J. (2010). Cancer-associated fibroblasts are positively correlated with metastatic potential of human gastric cancers. J. Exp. Clin. Cancer Res. *29*, 66.

Zucker,S., Doshi,K., and Cao,J. (2004). Measurement of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMP) in blood and urine: potential clinical applications. Adv. Clin. Chem. *38*, 37-85.