Loss of Functional Alpha-1-Antitrypsin and Heparin Cofactor II in Inflammation and Cancer

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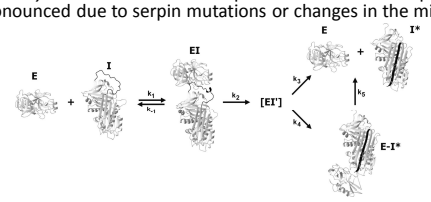
Introduction

Altered plasma levels of serpins have been reported in various cancer types, however their concentration is often measured by immunological methods (ELISA) rather than by inhibitory activity. The aggregate immunological assay combines both the intact and cleaved serpin forms. Cleaved serpins are the product of the *substrate pathway* in the bifurcated serpin mechanism. This pathway can become more pronounced due to serpin mutations or changes in the micro-environment.



Olson, S.T. and Gettins, P.G.W. (2011). Prog. Mol. Biol. Transl. Sci. 99, 185–240.
Proteinase **(E)** and serpin **(I)** form a reversible Michaelis complex **EI**. The proteinase cleaves the serpin in the reactive center loop (RCL), with formation of an acyl-enzyme intermediate **EI'**. The cleaved RCL, with proteinase attached, translocates 180° to form a covalent complex **(E–I*)**. If deacylation is completed prior to the inactivating proteinase distortion, cleaved serpin **(I*)** and regenerated free proteinase **(E)** are formed. Deacylation of **E–I*** can also slowly occur.

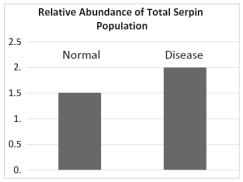
Methods - Results

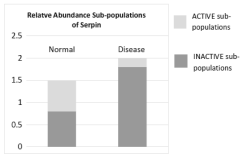
α1-Antitrypsin (AAT, SERPINA1) and heparin cofactor II (HCII, SERPIND1) respectively inactivate neutrophil elastase and thrombin.

- Increased antigen levels of AAT were reported in pancreatic cancer[1,2], and of HCII in non-small lung cell cancer[3].
- Sample preparation of control and pathological sera was performed by binding either to AlbuVoid^{R} beads that bind low abundance proteins but not albumin; and AlbuSorb^{R} beads that bind albumin and release low-abundance proteins in the flow-through.
- AlbuVoid^{R} was found to allow selective binding of serum AAT and HCII with intact RCL. The RCL-cleaved serpin fraction eluted in the flow-through.

LC – MS/MS

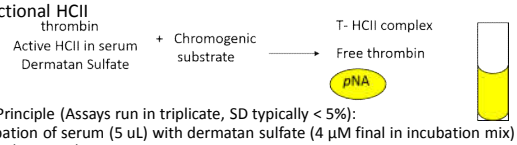
- Tryptic digestion of AlbuVoid-bound and eluted fractions of pooled controls and pooled pancreatic cancer patient samples
- Tandem mass tag labeling of peptides and single LC-MS/MS gradient run (3000 RSLCnano interfaced with Q Exactive HF Orbitrap), MS/MS scans of 20 most intense ions
- LC-MS of peptides indicated a predominance of peptides with **intact** Met358-Ser359 (AAT P1-P1') and Leu444-Ser445 (HCII P1-P1') in the **bound** fractions; and peptides with C-terminal Met/N-terminal Ser flanking (AAT), and with C-terminal Leu/N-terminal Ser flanking (HCII), representative of **cleaved** RCL, in the **flow-through** fractions
- Leu-Ser bonds are impervious to tryptic digestion, and Met-Ser is less sensitive than Arg-Ser
- Although total antigen levels of serpins may be higher in cancer and inflammation, the ratio cleaved/total serpin was observed to be higher in disease states



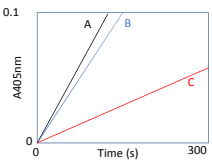


Assay of Functional Serpins in Serum

- Functional AAT – ongoing
- Functional HCII



Assay Principle (Assays run in triplicate, SD typically < 5%):
- Incubation of serum (5 uL) with dermatan sulfate (4 µM final in incubation mix) in assay buffer (< 100 µL)
- Start reaction by adding thrombin (0.13 µM final in incubation mix), total volume 100 µL. This amount of thrombin should be sufficient to trap all the HCII in serum in a covalent complex (normal serum value 2 to 2.5 µM, at 20x dilution is 0.100 – 0.125 µM)
- After incubation for 3 minutes, add buffer and chromogenic substrate to 1 mL and measure pNA absorbance at 405 nm, which reflects residual thrombin activity.



A. Rate for thrombin in buffer (reference value)
B. Rate for thrombin in serum, no dermatan sulfate. The uncatalyzed thrombin – HCII reaction is extremely slow, and the small decrease in thrombin activity reflects binding to other serum components (corrected reference)
C. Rate for residual thrombin activity after incubation with serum (containing active HCII) and DS.

The serum HCII concentration is calculated from B – C and dilution correction.

- Functional antithrombin

Assay Principle (Assays run in triplicate, SD typically < 5%):
- Incubation of serum (5 uL) with fondaparinux (145 µM final in incubation mix) in assay buffer (< 100 µL)
- Start reaction by adding factor Xa (0.1 µM final in incubation mix), total volume 100 µL. This amount of factor Xa should be sufficient to trap all the antithrombin in serum in a covalent complex (serum values are usually a bit lower than plasma, ~2 µM, at 20x dilution this is 0.100 µM)
- After incubation for 3 minutes, add buffer and chromogenic substrate to 1 mL and measure pNA absorbance at 405 nm, which reflects residual factor Xa activity.

Assay of Functional Serpins in Serum (*continued*)

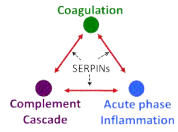
Sample Identifier	Active HCII	Active anti-thrombin
Breast cancer	0.061 mg/ml	0.054 mg/ml
Non-cancer Inflamm.	0.039 mg/ml	0.071 mg/ml
Normal/healthy	0.074 mg/ml	0.068 mg/ml
Normal/healthy	0.085 mg/ml	0.085 mg/ml
Normal/healthy	0.087 mg/ml	0.053 mg/ml
Lymphoma	0.037 mg/ml	0.058 mg/ml
Ovarian cancer	0.006 mg/ml	0.022 mg/ml

Consistent with the LC-MS/MS results, levels of *active* HCII with intact RCL were *significantly lower* in sera of patients with cancer and inflammation, compared to healthy individuals.
In this first cohort, no clear differences in antithrombin activity were seen between healthy and pathological samples.
AAT assays are ongoing, and a larger cohort is currently being analyzed. Results for AAT activity are expected to parallel those of HCII activity.

AAT and HCII are part of a cancer biomarker panel (Stroma Liquid Biopsy™), that combines critical components of coagulation, complement and acute phase inflammation[5,6]

The emphasis of current liquid biopsy platforms is on the tumor cell genome, however the supporting microenvironment also contributes to pathogenesis. The significance of the Stroma Liquid Biopsy™ pan-cancer profile lies in the observed dysregulation of interconnected, major proteolytic networks in the host. This dysregulation is often already obvious in early stages of disease. The biomarkers are either proteases, substrates or protease inhibitors.

Protein Conc. Range > 5 log measurable in 1 LC-MS Analysis					
Systemic Pathway	Protein Gene Name	Protein Description	Serum Conc. In normal/h ealthy	Spectral Intensities cancer relative to normal	Comments
Coagulation	PF4	Platelet Factor 4	10 ng/ml	↑	Severe, released from platelets
Coagulation	PPBP	Pro-platelet basic protein	5 µg/ml	↑	Severe, released from platelets, activates Neutrophils
Coagulation	TIMP1	Tissue inhibitor of metalloproteinases-1	100 ng/ml	↑	Severe
Coagulation	THBS1	Thrombospondin 1	200 ng/ml	↑	Released from platelets, multifunctional with some sequence and functional similarities to Complement regulating protein – Properdin below
Complement	C3	C3	1,500 µg/ml	↓	Complement cascade function & regulation is multi-faceted, Coagulation protein Thrombin activates C3
Complement	C4BPA	Complement Component 4 binding protein alpha	300 µg/ml	↓	Complement cascade function & regulation is multi-faceted
Complement	PROP	Properdin	25 µg/ml	↓	Released from Neutrophils, some sequence and functional similarities to coagulation protein THBS1
Acute-phase Inflammation	SAA2	Serum Amyloid 2	5 µg/ml	↑	Near limits of detection with current methods
Acute-phase Inflammation	ELANE	Neutrophil Elastase	250 ng/ml	↑	Near limits of detection with current methods
Acute-phase Inflammation	ECM1	Extracellular Matrix Protein 1	800 ng/ml	↑	Released from Platelet dense granules, severe in many chronic inflammatory conditions, might be rule in/out marker based on severity stratification
Acute-phase Inflammation	CMGA	Chromogranin A	-	↑	Only Lymphoma but severely differential from 5 primary tumors tested, below limits of detection for all others and all normals
SERPIN Function	SERPIN A1	Alpha-1-Antitrypsin	1,500 µg/ml	↓	Inhibits Neutrophil Elastase, and activated Protein C (a regulator of the coagulation cascade)
SERPIN Function	SERPIN D1	Heparin Cofactor II	60 µg/ml	↓	Inhibits extravascular Thrombin, Neutrophil Cathepsin G
SERPIN Function	SERPIN A3	Antichymotrypsin	300 µg/ml	inconclusive	Might be Tissue Specific? Complexes with Prostate Specific Antigen



The hypothesis is that there may be a common systemic response to many forms of cancer, regardless of primary tumor, stage or development of metastatic disease. This response is proposed to involve interconnected pathways of coagulation/hemostasis, acute-phase inflammation and the complement pathway. All these pathways are regulated through proteolysis and protease inhibition.

Conclusions and Future Work

- Selective, bead-based sample enrichment, combined with LC-MS/MS[4] has the potential of distinguishing between active and cleaved subpopulations of AAT and HCII in pooled sera of healthy individuals and of patients with pancreatic cancer. Tryptic digestion of AAT and HCII preserves the P1-P1' sequences in the RCL, and the method allows measuring the ratio of cleaved/total serpin.
- Preliminary results indicate a significant increase in the cleaved/total ratio in diseased samples, corresponding to a net decrease in inhibitory active serpin. This is in contrast with antigen-based measurements (ELISA) of total serpin[1-3], that indicate increased total serpin concentration regardless of inhibitory potential.
- Functional serpin assays of HCII indicated reduced inhibitory activity in serum samples of diseased individuals. AAT activity assays are ongoing, and a larger panel of sera is currently under study. Results for antithrombin were inconclusive and assays will also be performed on whole plasma to evaluate potential effects of sample preparation.
- AAT and HCII control the activities of the inflammatory proteases elastase and thrombin. Inflammation and tumorigenesis are proposed to be characterized by chronic exhaustion of inhibitory active serpins, and increased protease activity. Further analysis may include the extravascular space, as 60% of HCII is extra-vascular, and control of thrombin activation of PAR-1 and cleavage of Complement C3 may be of therapeutic advantage.

1. El-Akawi et al., World J Oncol 2013;4(2):83-86
2. Trichopoulos et al, Int. J. Cancer 1990; 45, 685-686
3. Liao et al., J Pathol 2015; 235: 50–64
4. Roy, Swapan, and Matthew Kuruc. "Methods to Monitor the Functional Subproteomes of SERPIN Protease Inhibitors." *Functional Proteomics*. Humana Press, New York, NY, 2019. 41-54.
5. Stroma Liquid Biopsy™ - Blood-based biomarkers to monitor stromal conditioning in cancer. Whitepaper February 2019. http://www.biotechsupportgroup.com/v/vspfiles/templates/257/pdf/StromaLiquidBiopsyWhitepaper022519.pdf
6. Roy, Swapan, Devjit Roy, and Matthew Kuruc. "Monitoring Dysregulated Serum Complement, Coagulation, and Acute-Phase Inflammation Sub-Proteomes Associated with Cancer." U.S. Patent Application No. 15/953,260.

Poster reprint first presented at The Serpins2019 Conference, September 19-22, 2019 in Sevilla, Spain