New Cancer Therapeutic Strategies are Proposed Based on Evidence from Stroma Liquid Biopsy™ Biomarkers

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Abstract

Tumors are more than simply a collection of immortalized cells as the supporting microenvironments or stroma also contributes to pathogenesis. Because of this, tumor characterization cannot be fully characterized solely through the analyses of the tumor cell genome – the current emphasis of liquid biopsy platforms. So because tumors are more than just a mass of proliferating cells, cancer progression must take into consideration the influence of the multiple cell types and networks of proteins dynamically interacting in active tumorigenesis. These are not simply passive bystanders. The unique significance of the Stroma Liquid Biopsy™ pan-cancer profile is that dysregulation was categorically intertwined with the most rudimentary needs of cancer: space, nutrients and immune evasion. Moreover, the changes within the 13 biomarker panel all occur within an interdependent network of cascading proteolytic events. Because proteolysis is irreversible, all species of life have evolved molecular regulatory systems to control aberrancies. The most distinguished is a protease inhibitory family of regulators known as SERPINs. Although SERPINs circulate in a variety of functional on/off sub-forms, conventionally they are observed and reported in aggregate (i.e., ELISA). As a result, their influence on disease is missed. Using assays that directly assess SERPIN function, rather than antigen presentation, we now have evidence that there is an imbalance of functional SERPIN sub-forms in the cancer population relative to that of a normal population. We highlight that two critical SERPIN nodes that regulate extravascular (though not necessarily intravascular) Thrombin inhibition are dysregulated in cancer. Therefore, specific targeting of extravascular Thrombin activity, or its consequential effects (C3 activation for one) are warranted as potential therapeutic modalities.

Introduction

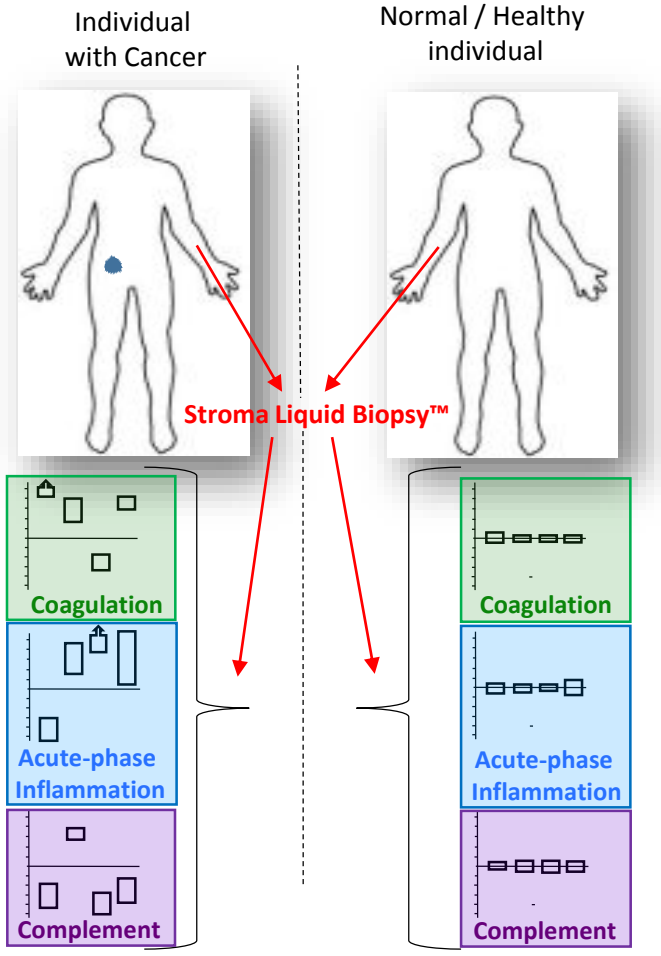
Is there a common systemic response to cancer?

We set out to answer whether there is a common blood response to most if not all cancers, regardless of primary tumor, stage, or metastatic disease.

Yes, the common systemic response is reflected in a biomarker pattern from "wounds that never heal" pathways including:

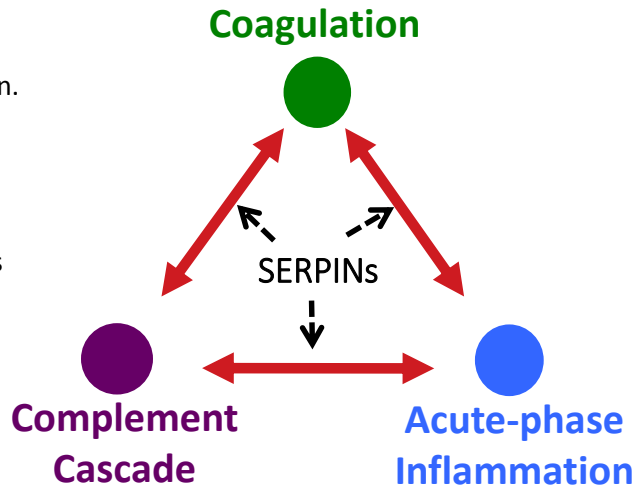
Coagulation
Acute-phase Inflammation
Complement

These pathways are all:
>systemically interconnected
>regulated through proteolytic post-translational modifications



In a recent whitepaper and disclosed in pending patent, we describe dysregulated serum Complement, Coagulation, and Acute-Phase Inflammation sub-proteomes associated with cancer[1,2]. This rewiring of the blood circuitry is measurable even at early stages of cancer, for many if not most primary tumors, forming the basis of intellectual property. So because tumors are more than just a mass of proliferating cells, cancer progression must take into consideration the influence of the multiple cell types and networks of proteins dynamically interacting in active tumorigenesis. These are not simply passive bystanders.

The unique significance of this pan-cancer profile is that this dysregulation was categorically intertwined with the most rudimentary needs of cancer: space, nutrients and immune evasion. Moreover, the changes within the 13 biomarker panel all occur within an interdependent network of cascading proteolytic events. Because proteolysis is irreversible, all species of life have evolved molecular regulatory systems to control aberrancies. The most distinguished is a protease inhibitory family of regulators known as SERPINs.

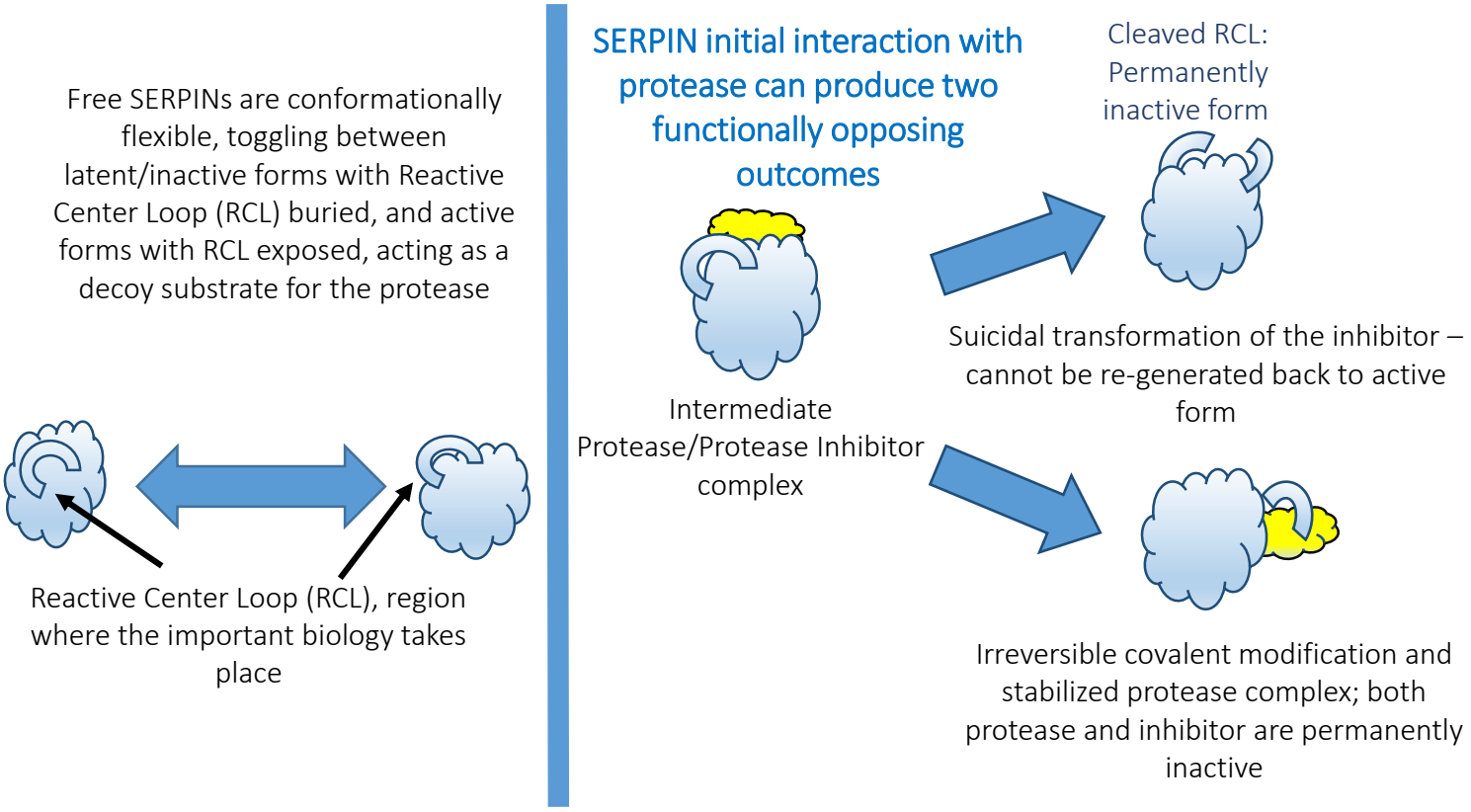


The Stroma Liquid Biopsy™ panel of biomarkers for cancer

Protein Conc. Range > 5 log measurable in 1 LC-MS Analysis					
Systemic Pathway	Protein Gene Name	Protein Description	Serum Conc. In normal/ healthy	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

Functional SERPIN Dysregulation

α-1-Antitrypsin (AAT, SERPINA1) and Heparin Cofactor II (SERPIND1) belong to the SERPIN gene family of suicidal protease inhibitors. To quantify their functions, their suicidal mechanism of action demands much different proteomic accounting methods than if their functions were simply stoichiometric, as would be the case if they were not suicidal-type inhibitors. This is illustrated here.



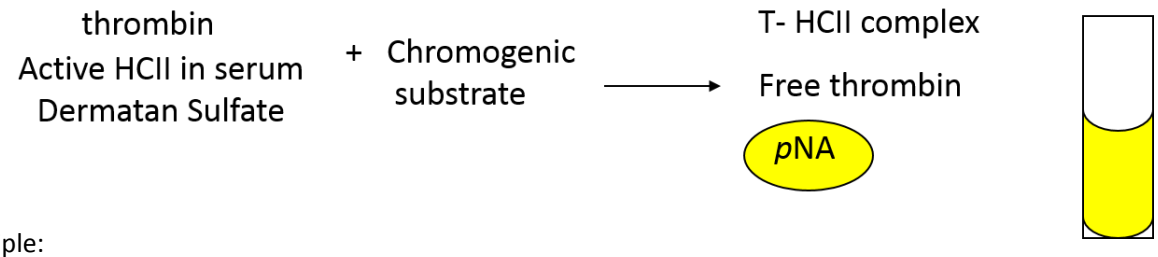
We have developed methods to make this critical distinction by measuring Liquid Chromatography-Mass Spectrometry (LC-MS) traceable peptide features within the RCL region of Serpins[3]. These distinct sub-populations we now can measure and observe as:

> "ACTIVE" or [+] all Serpin sub-populations that report as having transient inhibitory potential

> "INACTIVE" or [-] all Serpin sub-populations that report a suicidal transformation with permanent loss of inhibitory function.

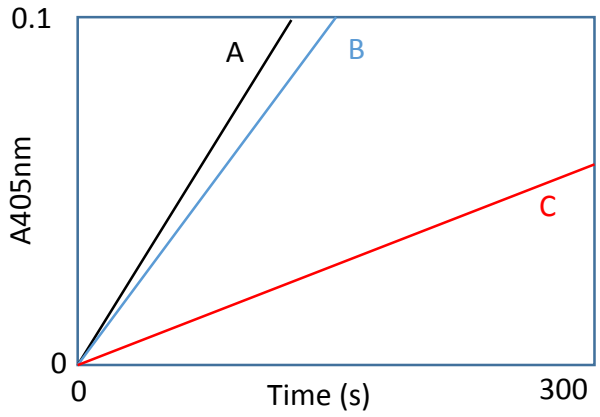
This is in contrast to the more conventional quantitative approaches which only observe the total population of Serpins by antigen presentation (i.e., ELISA). Such measurements not only discount the significance of whether the Serpin is functional or not, but can lead to misinterpretation of pathway effector mechanisms. Such is the case in cancer, when Serpins are observed by total populations rather than functional sub-populations. Unlike in many previous reports on AAT populations in cancer, we observe that in cancer sera, there is a decline in the abundancy of [+] AAT sub-populations[1,2]. The same is true with the other Serpin in our Stroma Liquid Biopsy™ panel, SERPIND1, otherwise known as Heparin Cofactor II. To further validate that LC-MS spectral correlate with the inhibitory potential, we investigated the use of inhibitor specific reporting by functional enzyme assays.

Measurement of HCII inhibitory activity in human serum samples



Assay Principle:

- Incubation of serum (5 uL) with dermatan sulfate (DS, 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.

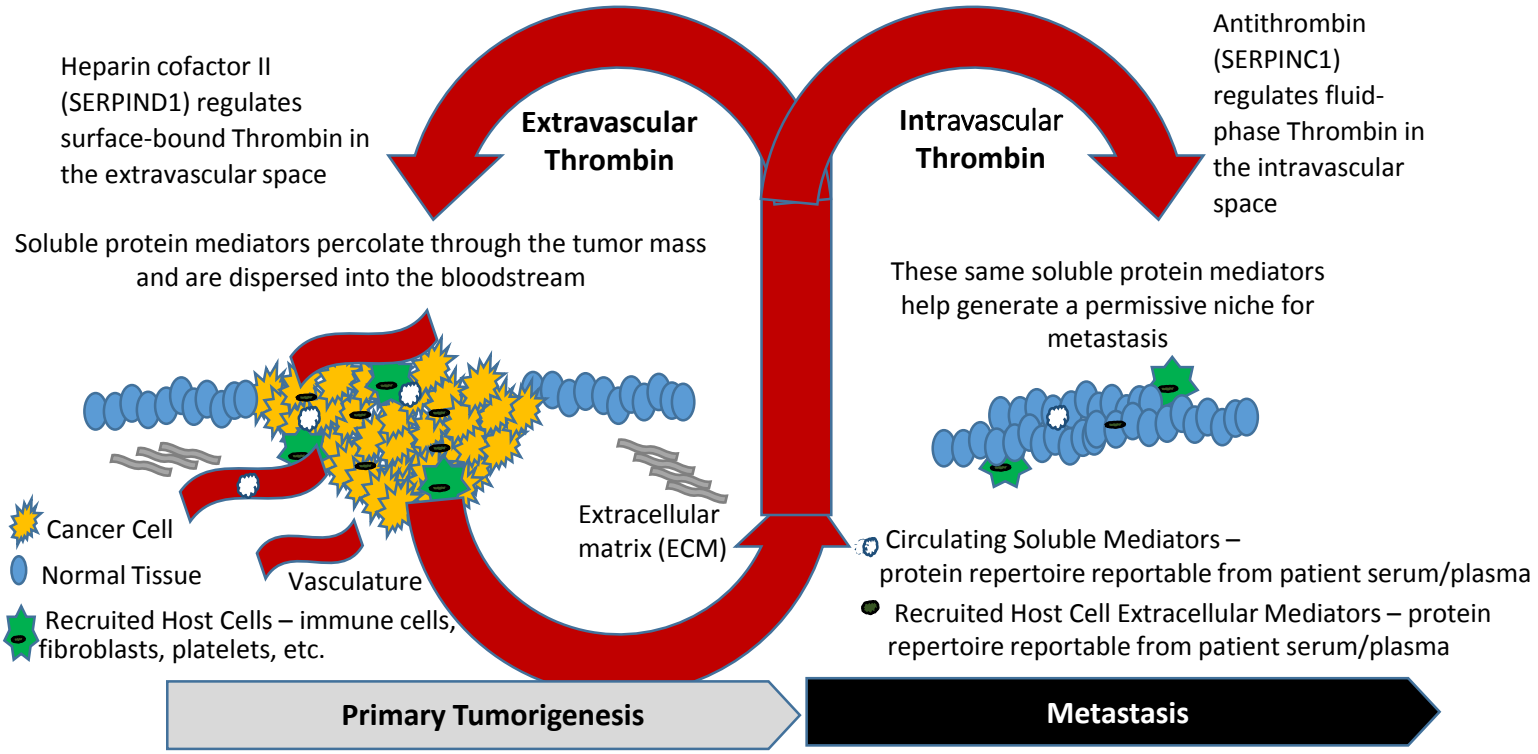
Measurement of antithrombin inhibitory activity in human serum samples

Assay Principle:

- Incubation of serum (5 uL) with the heparin pentasaccharide - 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 (Spectrozyme fXa, American Diagnostica) to 1 mL and measure pNA absorbance at 405 nm, which reflects residual factor Xa activity. Assays run in duplicate, SD typically < 5%.

Sample Identifier	Active HCII (mg/ml)	Rel. LC-MS spectral intensity RCL cleaved[3]	Active anti-thrombin (mg/ml)
Normal/healthy	0.074	1.0	0.068
Normal/healthy	0.085	0.9	0.085
Normal/healthy	0.087	1.5	0.053
Normal Average	0.081	1.1	0.069
Lymphoma	0.037	0.3	0.058
Ovarian cancer	0.006	0.2	0.022
Breast cancer	0.061	0.6	0.054

Two data sets from different analytical platforms align and support that there are inflammatory proteases in tumorigenesis that are not sufficiently regulated due to chronic exhaustion of ACTIVE proteoforms of SERPIN protease inhibitors!



Conclusions

A variety of anticoagulants have been clinically tested, with some benefits especially for very early stage cancers. From our data and model, we can solicit that extravascular (though not necessarily intravascular) Thrombin inhibition is insufficiently controlled in cancer. This reinforces the notion to neutralize Protease Activated Receptor (i.e., PAR1) activation as a therapeutic strategy. In an ideal personalized setting, inhibiting Thrombin's cleavage of Complement C3 to its activated fragments – C3a (anaphylatoxin) & C3b (Initiator of MAC - Membrane Attack Complex), might also be advantageous. Thus, specific allosteric modulation of extravascular Thrombin is warranted as a therapeutic modality.

The pleiotropic nature of Serpins, especially α-1-Antitrypsin (SERPINA1) and Heparin Cofactor II (SERPIND1), serve as critical regulating nodes in the tumor and systemic microenvironments promoting carcinogenesis. Reactive center peptides derived from Serpins have shown potential for immunomodulatory functions, and a precise tuning of Serpin stromal modulation might be possible. This will greatly impact the management and treatment of cancer owing to the systemic metastatic potentiation derived from coagulation activation.

References

1. 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
2. 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.
3. 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.

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