Protein Signature of Lung Cancer Tissues

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Abstract

Lung cancer remains the most common cause of cancer-related mortality. We applied a highly multiplexed proteomic technology (SOMAscan) to compare protein expression signatures of non small-cell lung cancer (NSCLC) tissues with healthy adjacent and distant tissues from surgical resections. In this first report of SOMAscan applied to tissues, we highlight 36 proteins that exhibit the largest expression differences between matched tumor and non-tumor tissues. The concentrations of twenty proteins increased and sixteen decreased in tumor tissue, thirteen of which are novel for NSCLC. NSCLC tissue biomarkers identified here overlap with a core set identified in a large serum-based NSCLC study with SOMAscan. We show that large-scale comparative analysis of protein expression can be used to develop novel histochemical probes. As expected, relative differences in protein expression are greater in tissues than in serum. The combined results from tissue and serum present the most extensive view to date of the complex changes in NSCLC protein expression and provide important implications for diagnosis and treatment.

Introduction

Progression from healthy state to disease is accompanied by changes in protein expression in affected tissues. Comparative interrogation of the human proteome in healthy and diseased tissues can offer insights into the biology of disease and lead to discovery of new biomarkers for diagnostics, new targets for therapeutic intervention, and identification of patients most likely to benefit from targeted treatment. In particular, new diagnostics for early detection of lung cancer are urgently needed. For the purposes of treatment and prognosis, lung cancer is classified pathologically as either small cell (15%) or non-small cell (85%). Lung cancer is the leading cause of cancer deaths, largely because 84% of cases are diagnosed at an advanced stage, with a five-year survival rate of less than 15% [1–3]. Worldwide in 2008, 1.5 million people were diagnosed and 1.3 million died – a survival rate unchanged since 1960 [4]. However, patients diagnosed with NSCLC at an early stage and treated surgically to remove their tumors experience an 86% five-year survival [1,2].

We recently developed a novel affinity-based proteomic technology for biomarker discovery that currently measures over 1,000 proteins from small sample volumes of plasma or serum (e.g., ~10 μL of plasma) with low limits of detection (median value of 300 fM), 7 logs of overall dynamic range (~30 fM – 1 μM, using sample dilution), and 5% median coefficient of variation [5]. This technology, called SOMAscan, is enabled by SOMAmers (Slow Off-rate Modified Aptamers), a new class of protein binding reagents that contain chemically modified nucleotides, which greatly expand the physicochemical diversity of the nucleic acid libraries. Such modifications introduce functional groups that are often found in protein-protein interaction, antibody-antigen interactions, and interactions between small-molecule drugs with their protein targets, but are absent in natural nucleic acids. These modifications are compatible with the SELEX (Systematic Evolution of Ligands by EXponential Enrichment) process used to create SOMAmers as well as standard DNA methods including PCR and hybridization. Overall, the use of these modifications expands the range of possible targets for SELEX, results in improved binding properties, and facilitates selection of SOMAmers with slow dissociation rates [5].

SOMAscan is a highly multiplexed platform for quantitatively measuring proteins in complex matrices such as plasma or serum in which a signature of protein concentrations is transformed into a corresponding DNA signature, which is then quantified on a commercial DNA microarray platform [5]. Briefly, equilibrium binding between a mixture of SOMAmers and proteins is achieved in solution, followed by removal of unbound species by successive bead-based immobilization steps accompanied with extensive washing. High specificity, already an intrinsic feature of SOMAmers, is additionally enhanced with the inclusion of dextran sulfate during binding and washing steps. Dextran sulfate, which like nucleic acids is a polyanion, is effective because cognate SOMAmers are more kinetically stable than non-specific complexes. At the end of the assay, specific SOMAmers can be eluted under denaturing conditions, hybridized on commercially available microarrays, and directly quantified through a fluorophore covalently coupled to the SOMAmer. In
essence, the assay takes advantage of the dual nature of SOMAmers as both folded binding entities with defined shapes and unique nucleic acid sequences recognizable by specific hybridization probes. The utility of this assay has been shown previously in simultaneous measurements of large numbers of proteins ranging from low picomolar to high micromolar concentration in plasma and serum and clinical biomarker studies of chronic kidney disease and lung cancer [5,6].

Results

Proteomic analysis of NSCLC surgical resections

In this report, we performed large-scale protein expression analysis of homogenized lung tissue samples from surgical resections obtained from eight non-small cell lung cancer (NSCLC) patients. All NSCLC patients were smokers, ranging in age from 47 to 75 years old and diagnosed with pathology-confirmed NSCLC stages IA through IIIB (Table 1). We obtained three samples from each resection: tumor tissue sample, adjacent non-tumor tissue (within 1 cm of the tumor) and distant uninvolved lung tissue (furthest edge of the resection from the tumor). Care was taken to preserve the integrity of the tissue, with all samples being frozen within 5–10 minutes of excision. Total protein concentration was adjusted and normalized in each homogenate for proteomic profiling followed by analysis on our biomarker discovery array to measure the concentrations of 820 human proteins as recently described [5].

These protein concentration measurements, expressed as relative fluorescence units (RFU), allow large-scale comparisons of protein signatures among samples (Fig. 1). We first compared the protein expression levels between the adjacent and distant tissue samples for each patient (Fig. 1A). Overall, the signals generated by most analytes were similar in adjacent and distant tissue. In this comparison, only one analyte (fibrinogen) exhibited more than a two-fold difference between the two control samples. Fibrinogen concentration was higher in adjacent non-tumor tissue than distant non-tumor tissue. Fibrinogen is the soluble precursor of fibrin, which is converted by thrombin during coagulation. Fibrin deposits occur within adjacent stroma of most tumors, primarily in the extracellular matrix (ECM) where fibrin and other ECM proteins promote and support tumor growth processes including, cell proliferation, adhesion, invasion, migration, and angiogenesis [7].

In contrast, comparison of tumor tissues with non-tumor tissue (adjacent or distant) identified 11 (1.3%) proteins with greater than four-fold differences and 53 (6.1%) proteins with greater than two-fold differences (Figs. 1B and 1C). The remaining (93.9%) proteins showed relatively small differences between tumor and non-tumor tissue. Some proteins were substantially suppressed while others were elevated in tumor tissues compared to adjacent or distant tissues. Differential expression of proteins between adjacent and tumor tissue, or between distal and tumor tissue, was similar overall. Changes in between tumor and distal tissue were generally somewhat larger compared to tumor and adjacent tissue (Fig. 1), which demonstrates that most observed protein changes are specific to the local tumor environment. Figure 2 shows a heat map depiction of the results. There was a trend of protein changes reflecting pathologic stage, which may indicate that protein expression correlates with disease burden. Given the small sample size, correlations with histological classification could not be decoupled from stage.

Biomarker identification

To identify potential NSCLC tissue biomarkers, we looked for analytes with the largest change in protein expression levels between tumor, adjacent, and distant tissue samples. Here we highlight thirty-six proteins with the largest mean fold-change in protein expression between tumor and non-tumor tissue samples (Fig. 3, Table 2). We tested the significance of these changes with the Mann Whitney test and required a p-value of 0.05 after correcting for multiple tests (false discovery rate cutoff of q<0.05).

Although the number of samples we used for this study was relatively small, the study consisted of paired tumor and non-tumor tissue samples from each individual. This provides more power to identify changes within an individual and eliminates the population variance associated with cross-sectional study designs. The availability of appropriately chosen reference samples is increasingly recognized as a crucially important component in biomarker discovery research [8–10]. Finally, we assessed reproducibility of this new method by analyzing triplicate samples of tumor and non-tumor tissue resections for two subjects in this study and found a 4.5% median CV between triplicate

Table 1. Patient demographics, resection location and tumor types for the eight NSCLC analyzed samples.

| Age | Sex | Smoking History | Location          | Stage                          | Tissue Dx                              |
|-----|-----|-----------------|-------------------|-------------------------------|----------------------------------------|
| 47  | F   | Smoker          | Left Upper Lobe   | pT3pN1pMx stage IIIA          | Poorly differentiated non-small cell CA with focal squamous differentiation |
| 73  | F   | Smoker          | Left Lower Lobe   | pT2pN0pMx stage IB            | Poorly differentiated squamous cell carcinoma |
| 48  | M   | Smoker          | Right Upper Lobe  | pT2pN1pMx stage IIIA          | Poorly differentiated squamous cell carcinoma |
| 60  | F   | Smoker          | Left Upper Lobe   | T4 N1 M0 stage IIIIB - note T4 distinction based on clinical lung collapse; tumor was pT2 by size criteria | Poorly differentiated squamous cell carcinoma |
| 51  | F   | Smoker          | Right Upper Lobe  | pT2pN0pMx stage IB            | Moderately differentiated adenocarcinoma |
| 71  | F   | Smoker          | Right Upper Lobe  | pT2pN0pMx stage IB            | Well differentiated adenocarcinoma     |
| 75  | F   | Smoker          | Right Lower Lobe  | pT1N0Mx Stage IA              | Well differentiated adenocarcinoma     |
| 73  | M   | Smoker          | Left Upper Lobe   | pT1bN0Mx Stage IA             | Atypical carcinoid tumor (i.e. neuroendocrine, IHC positive for chromogranin)       |

DOI: 10.1371/journal.pone.0035157.t001
measurements for the 820 proteins measured (Fig. 4) and Spearman correlation coefficients \(0.99\) (partially inflated by the large RFU range measured). Triplicate measurements for the 36 proteins with largest mean-fold differences between tumor and non-tumor tissue are plotted in Fig. 5.

High-content proteomic analysis of biological samples enabled by our multiplexed assay allows unbiased discovery of disease-related proteins. To date, we have conducted several blood-based clinical biomarker studies of human diseases, including lung cancer [6] and chronic kidney disease [5]. These studies have identified novel potential disease biomarkers as well as biomarkers that have been reported previously. The current study follows this trend. About one-third (13/36) of the potential NSCLC tissue biomarkers identified here are novel, to the best of our knowledge. The remaining two-thirds (23/36) have been reported previously as differentially expressed proteins or genes in NSCLC tumor tissue (Table 2). Novelty was determined by performing literature searches in Pubmed and on the internet using the potential biomarkers’ gene names and protein aliases as identified by UniProt.

**Figure 1. Relative changes in protein expression for 820 proteins from eight NSCLC resection samples.** Signal differences between adjacent and distant tissue (panel A), tumor and adjacent tissue (panel B) and tumor and distant tissue (panel C) are expressed as log₂ median ratios. The dotted line represents two-fold change (log₂ = 1).

doi:10.1371/journal.pone.0035157.g001
The potential biomarkers can be classified broadly into four biological processes associated with important hallmarks of tumor biology [11] as shown in Table 3: 1) angiogenesis, 2) growth and metabolism, 3) inflammation and apoptosis, and 4) invasion and metastasis. Admittedly, these are convenient but inexact classifications that approximate a highly complex and dynamic system in which these molecules often play multiple and nuanced roles. Therefore, the specific state of a given system ultimately affects the expression and function of any particular molecule. Our understanding of the biological underpinnings of these systems is far from complete. With the SOMAscan platform, we are beginning to explore the quantitative expression of large numbers of proteins in various tissues and disease processes. These data provide new coordinates to help map the dynamics of these systems, which in turn will provide a more complete understanding of the biology of this disease. The results from the current study provide a new perspective on NSCLC tumor biology, with both familiar and new elements.

Angiogenesis

Angiogenesis drives growth of new blood vessels to support tumor growth and metabolism. The regulation of angiogenesis is a complex biological phenomenon controlled by both positive and negative signals [11]. Among the potential NSCLC tissue biomarkers identified in this study (Fig. 3) were well known positive and negative angiogenesis regulators, all of which have been observed previously in NSCLC tumor tissue [12–16]. These include the prototypic angiogenesis inducer VEGF and inhibitors endostatin and thrombospondin-1 (TSP-1). VEGF is a powerful growth factor that promotes new blood vessel growth; VEGF was strongly up-regulated in NSCLC tumor tissue, consistent with previous observations [12], including our study of serum samples from NSCLC patients [6]. It is worth noting that VEGF was originally discovered as tumor cell-secreted vascular permeability factor (VPF) that increased the leakiness of tumor-associated blood vessels to large molecules, such as fibrinogen, that are normally confined to plasma [17]. This activity may have profound effects on the composition of proteins associated with tumor tissue.
Endostatin is a proteolytic fragment of collagen XVIII and a strong inhibitor of endothelial cell proliferation and angiogenesis [13]. TSP-1 and the related TSP-2 were substantially up-regulated in NSCLC tumor tissue. TSP-1 and TSP-2 are extracellular matrix proteins with complex, context-dependent effects modulated through a variety of interactions with cell-surface receptors, growth factors, cytokines, matrix metalloproteinases, and other molecules. Archetypically in model systems, TSP-1 and TSP-2 inhibit angiogenesis by inhibiting endothelial cell proliferation through the CD47 receptor (not measured in this study) and inducing endothelial cell apoptosis through the CD36 receptor. There is also evidence for proangiogenic influences for TSP-1 and TSP-2 [18]. Finally, reported TSP-1 and TSP-2 relative and absolute expression levels in NSCLC tissue vary [16,19–21] likely

Table 2. SOMAscan protein expression differences expressed as log2 ratio of the signal in tumor to non-tumor tissue samples for proteins with the largest mean-fold change.

| Protein Target | Adjacent | Distant | NSCLC Tissue Expression Changes Reported in Literature |
|----------------|----------|---------|-------------------------------------------------------|
| Activin A      | 1.16     | 0.88    | Protein [39]; Gene [39–41]                           |
| Adiponectin    | −0.99    | −1.00   | None                     |
| BCA-1          | 1.91     | 2.24    | None                     |
| Biglycan       | −1.88    | −1.14   | None                     |
| Cadherin-1     | 1.33     | 1.21    | Protein [42–45]                           |
| Carbonic anhydrase III | −2.22 | −2.13 | None                     |
| Caspase-3      | 0.95     | 0.98    | Protein [46,47]; Gene [41,48]               |
| Catalase       | −1.03    | −0.93   | Protein [49,50]; Gene [50,51]               |
| CD36           | −0.91    | −1.24   | Gene [52]                     |
| CXCL16, soluble| −0.93    | −1.00   | None                     |
| Endostatin     | −1.03    | −1.12   | Protein [13,14,53]               |
| ESAM           | −1.52    | −1.81   | Protein [54]                     |
| Fibronectin    | 1.92     | 1.58    | None                     |
| IGFBP-2        | 2.42     | 2.58    | Gene [55,56]                     |
| IGFBP-5        | 2.45     | 1.77    | Gene [56,57]                     |
| IGFBP-7        | 1.52     | 0.47    | Protein [58,59]                     |
| IL-8           | 1.80     | 1.76    | Protein [60]; Gene [52,60,61]         |
| Insulysin      | 0.78     | 1.10    | None                     |
| MAPK13         | 1.28     | 0.89    | None                     |
| MMP-7          | 1.91     | 2.11    | Protein [26,62]; Gene [52,63]         |
| MMP-12         | 3.53     | 4.19    | Protein[26,27]; Gene [27,41,51,64]         |
| MRC1           | −1.36    | −1.96   | None                     |
| NAGK           | 0.83     | 1.24    | None                     |
| NAP-2          | −1.11    | −1.44   | Gene [41]                     |
| P-Selectin     | −1.57    | −1.78   | None                     |
| SLPI           | −1.77    | −0.85   | Gene [51]                     |
| sRAGE          | −5.77    | −5.44   | Protein [25,65]; Gene [25,41,51,65]         |
| Thrombospondin-1| 1.70   | 1.23    | Protein [20]; Gene [40,52]               |
| Thrombospondin-2| 1.80   | 1.93    | Protein[66]; Gene [40,52,66]            |
| TrATPase       | −2.26    | −2.87   | None                     |
| Tryptase β-2   | −2.68    | −2.24   | Protein [67]                     |
| uPA            | 1.08     | 0.94    | Protein [68]; Gene [52,64,66,69]         |
| URB            | 2.11     | 1.41    | None                     |
| VEGF           | 1.88     | 1.34    | Protein [12,14]; Gene [15,41,51]         |
| vWF            | −0.98    | −1.18   | Gene [41,51]                     |
| YES            | 0.85     | 1.02    | Gene [70,71]                     |
Inflammation and Apoptosis

Inflammation and apoptosis are hallmarks of cancer biology, and we find a number of potential biomarkers associated with these processes that have been associated previously with NSCLC (Table 2). We found caspase-3 concentrations higher in NSCLC tumor tissue. Caspase-3 has been associated with metastasis [24]. Another notable example is soluble receptor for advanced glycation end-products sRAGE, which has been reported to be dramatically down-regulated in NSCLC tissue [21,25]. This finding is consistent with our measurement, in which sRAGE had the largest observed change for proteins that are lower in tumor than in non-malignant tissue. One hypothesis is that RAGE plays a role in epithelial organization, and decreased levels of RAGE in lung tumors may contribute to loss of epithelial tissue structure, potentially leading to malignant transformation [25]. Several chemokines, such as BCA-1, CXCL16, IL-8, and NAP-2, are altered in our study, consistent with the hypothesis that invasion of tumors with cells from the innate and adaptive arms of the immune system provide bioactive molecules that affect proliferative and angiogenic signals [11].

Invasion and Metastasis

The largest group of potential biomarkers contains proteins that function in cell-cell and cell-matrix interactions and are involved in invasion and metastasis. Many have been previously reported to be associated with NSCLC. Most notable are two of the matrix metalloproteases, MMP-7 and MMP-12, which contribute to proteolytic degradation of extracellular matrix components and processing of substrates such as growth factors. For example, the major substrate for MMP-12 is elastin. Such processes are well known to play a role in creating tumor microenvironments. We observed MMP-7 and MMP-12 up-regulated in NSCLC tissue, which is consistent with similar study that used antibody-based measurements [26]. The over-expression of MMP-7 and MMP-12 has been associated with poor prognosis in NSCLC [26]. MMP-12 levels have been correlated with local recurrence and metastatic disease [26]. It is interesting to note that two of the eight subjects studied had normal levels of MMP-12, whereas the other six had 15–50-fold elevation of MMP-12 in tumor tissue compared to non-tumor tissue.

SOMAmers as histochemistry probes to NSCLC biomarkers

Understanding the differences in protein expression between tumor and non-tumor tissues can identify novel histochemistry targets. This approach was used previously with MMP-12 and others [27]. Such probes can enable more precise molecular characterization of tumors and their effects on the surrounding stroma. We have previously demonstrated that fluorophore-labeled SOMAmers confer rapid and selective histochemical staining in frozen tissue sections [28]. Here we examined tissue staining by several of the SOMAmers that were identified as biomarkers in our analysis of tissue homogenates. Frozen tissue sections were cut from the same tumor resections used for biomarker discovery. For example, TSP-2 staining with a fluorophore-labeled SOMAmer in tumor tissue was striking and localized predominantly in areas of fibrous stromal scarring (Fig. 6A), but such staining was largely absent in normal tissue (Fig. 6B). This is consistent with the reported role of TSP-2 in matrix modulation [18,29]. In contrast, in normal lung tissue, the macrophage mannose receptor (MRC1) SOMAmer staining localized to the surface of alveolar macrophages (Fig. 6D) as expected for this target [30]. Tumor tissue samples, which lack alveoli, showed little MRC1 staining (Fig. 6C). Figure 6E demonstrates MRC1 SOMAmer staining performed concurrently with antibody-based immunofluorescence for other targets (cytokeratins and CD31), indicating the feasibility of multiplexing SOMAmers and antibody reagents in histologic studies. Tissue staining with SOMAmers was thus consistent with the homogeneate profiles, in which TSP-2 was elevated and MRC1 was decreased in tumor versus healthy tissue. We confirmed the SOMAmer staining patterns of TSP-2 and MRC1 with antibodies...
Figure 7. Congruence between histochemical staining results with the direction of change in protein expression between tumor and healthy tissue homogenates provides additional evidence that the identified biomarkers are associated with disease. Large-scale proteomic comparison between tissues described here is also a powerful method for identifying novel histochemical probes.

Some general caveats related to discovery of potential NSCLC biomarkers are worth noting. First, the fact that a protein is associated with tumor tissue need not mean that it is specific for tumor tissue. For example, inflammation, extracellular matrix remodeling, hypoxia, and tissue necrosis accompanies tumor progression but also many other non-malignant conditions such as injury, wound healing or infection. Second, biomarkers we identified could reflect a difference in the ratio of cell types that constitute a tumor sample compared to that of the normal lung tissue. For example, if tumor tissue consists of cancer cell overgrowth, some of the biomarkers are expected to be specific for that cell type (in this case, epithelial cells), transformed or not. Similarly, if a tumor tissue sample is either more or less vascularized than the surrounding normal tissue, a change in the...
expression of endothelial cell-specific proteins may be observed. Indeed, we observed significantly lower concentrations of ESAM, a protein specific to endothelial cells, compared to matched, non-tumor tissue. We confirmed this histochemically, as shown in Figure 8, where we measured 35-fold more ESAM-positive endothelial cells in distant non-tumor tissue compared to tumor tissue. Finally, SOMAmers, like all affinity reagents, bind and recognize specific epitopes of target proteins generally in a conformation-dependent manner, and any particular measurement reflects the availability of that epitope.

Comparison of NSCLC tissue and serum biomarkers

We have recently completed a NSCLC study [6] in which we analyzed 1,326 serum samples from four independent clinical study centers using the same proteomic platform and a protein menu nearly identical to the that used for tissue (813/820 proteins). The study included patients diagnosed with pathologic or clinical stage I-III NSCLC and a control population with a history of long-term tobacco use, including active smokers and ex-smokers with at least 10 pack-years of cigarette smoking. Taking extensive precautions to account for pre-analytic variables, we identified 44 candidate biomarkers, and developed a 12-protein panel that distinguished NSCLC from controls with 91% sensitivity and 84% specificity in a training set, and 89% sensitivity and 83% specificity in a blinded, independent verification set. The availability of this database allows us to compare changes in protein expression in tissue and serum from NSCLC patients.

While the methodology used to analyze the protein profiles in samples from these two NSCLC studies is the same, some significant differences are worth noting. First, serum pre-analytic variability was observed between study centers, perhaps masking some cancer biomarkers [6]. Second, in the NSCLC tissue study reported here, each tumor sample has its own control tissue (adjacent and distant non-tumor), whereas the larger NSCLC serum study by necessity is composed of case and control samples from different individuals. Nevertheless, differential expression of proteins in sera of NSCLC patients relative to cancer-free controls compared with that of NSCLC tissue samples yields useful insights (Fig. 9, Table 4). The most striking observation is that relative changes in protein expression are greater in tissues than in serum. This result could be expected since tumor tissue is the source of the changes in protein expression that is then, even if fully released into circulation, diluted many-fold into total volume of blood. This trend is evident in the elongated distribution of data points along the x-axis in Figure 9 in which axes are drawn on the same scale to illustrate this point. Eleven of the analytes shown in Figure 3 as altered in tumor tissue were also differentially expressed in sera from NSCLC patients vs. controls (filled red circles in Fig. 9). It is worth noting that our published NSCLC serum study [6] did not measure MMP-12, which this study identified as a top tissue biomarker. In subsequent NSCLC serum

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Table 3. Categorization of NSCLC tissue biomarkers into biological major processes.

| Angiogenesis | Growth and Metabolism | Inflammation & Apoptosis | Invasion, Metastasis (ECM) |
|--------------|-----------------------|--------------------------|---------------------------|
| VEGF         | Adiponectin*          | Activin A                | Biglycan*                 |
| Endostatin   | Carbonic anhydrase III*| BCA-1*                  | Cadherin-1                |
| Thrombospondin-1 | IGFBP-2      | Catalase             | CD36                      |
| Thrombospondin-2 | IGFBP-5       | CXCL16, soluble*     | ESAM                      |
| Insulysin*   | IL-8                   | Fibronectin*          | Auxin                    |
| NAGK*        | NAP-2                  | MMP-12                 | P-Selectin*               |
| TrATPase*    | sRAGE                 | YES                     |                           |
| Tryptase b-2 | SLPI                   |                          |                           |
| MAPK13*      | uPA                    |                          |                           |
| Caspase-3    | Thrombospondin-1        |                          |                           |
| Thrombospondin-2 |             |                          |                           |

*Novel NSCLC Biomarker.

doi:10.1371/journal.pone.0035157.t003

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Figure 6. SOMAmer histochemistry on frozen tissue sections for selected biomarkers detected in this study. (A) Thrombospondin-2 SOMAmer (red) staining the fibrocollagenous matrix surrounding a tumor nest. (B) Corresponding normal lung specimen stained with Thrombospondin-2 SOMAmer (red). (C) Macrophage mannose receptor SOMAmer (red) staining scattered macrophages in a lung adenocarcinoma. (D) Macrophage Mannose Receptor SOMAmer (red) staining numerous alveolar macrophages in a section of normal lung parenchyma. (E) Multicolor image highlighting the cytomorphologic distribution of macrophage mannose receptor SOMAmer staining: Green = Cytokeratin (AE1/AE3 antibody), Red = CD31 (EP3095 Antibody), and Orange = SOMAmer. All nuclei in this figure are counterstained with DAPI.

doi:10.1371/journal.pone.0035157.g006
studies, MMP-12 was measured and we found it was also a top serum biomarker with a KS-distance of 0.42 (Table 2). This suggests that elevated serum MMP-12 directly reflects NSCLC tumor biology. Most of the other biomarkers common to tissue and serum also change in the same direction, but a few do not. Local concentrations of proteins in a tissue homogenate clearly need not correlate with circulating levels of the proteins and inverse correlations may provide clues regarding the redistribution of certain biomarkers in diseased versus normal tissues.

Discussion

The discovery of novel biomarkers with demonstrable diagnostic or clinical utility has been a considerable challenge in recent years [8]. The reasons for this include: the omnipresence of preanalytical and analytical artifacts, unavailability of suitable healthy-state controls, issues related to study designs, and the difficulty of detecting small changes in protein levels at very low concentrations. This challenge is especially pronounced with cancer biomarkers where the objective is often to find biomarkers of a tiny malignancy in the blood of a relatively large human body at an early stage.

The recently completed National Lung Screening Trial (NLST) reported a significant mortality benefit of screening for NSCLC with low dose CT and detecting early stage disease in a high risk population [31]. However, the high false positive rate results in a low (4%) positive predictive value (PPV) and potential harm from over diagnosis and unnecessary treatment. Complementary biomarkers that would either identify individuals who would most benefit from CT screening or improve the PPV value of imaging are in the research phase but are not yet in clinical use. Both miRNA and proteomic signatures in tissue and plasma have been reported [32,33]. Advances in understanding the molecular origins of NSCLC are beginning to guide the development of targeted therapies [34]. As reported by Sequist, approximately half of NSCLC tumors have known driver mutations, 22% of which are candidates for molecular targeted therapy [35].

One way to improve the chances of discovering true cancer biomarkers is to measure protein concentrations in both the source of the disease, such as tumor tissue, as well as from the circulation. Such combined results can support the validity of potential biomarkers and separate them from experimental artifacts. In this report, we have demonstrated that this is possible with our highly multiplexed and sensitive proteomic assay. We have shown that tissues, like plasma or serum, are also amenable to SOMAmer screen, and the resulting comparative analysis of protein expression in NSCLC tumor tissues with surrounding healthy lung tissues offers
a complement to our existing dataset of potential NSCLC biomarkers identified from serum samples. In our case, one third of the thirty-six tissue biomarkers reported here (BCA-1, cadherin-1, catalase, endostatin, IGFBP-2, MRC1, MMP-7, MAPK-13, NAGK, VEGF and YES) have been previously identified in serum [6]. Taken together, these data contribute to further understanding of the complexity of changes accompanying NSCLC and provide us with additional potential biomarkers for the early detection of this deadly disease.

Materials and Methods

This work was performed on deidentified samples that would have been disposed of if not used for research. This research thus qualified as a minimal risk to patients and their privacy, and approval for the use of these specimens with a waiver of consent was granted by the University of Washington’s Institutional Review Board.

Homogenate Preparation

All tissue samples for proteomic analysis were obtained by freezing the tissue within 5–10 minutes of excision during surgery and after placing the tissues in OCT medium (10.24% polyvinyl alcohol, 4.26% polyethylene glycol, and 85.5% non-reactive ingredients). Three samples were obtained from each resection: tumor tissue sample, adjacent healthy tissue (within 1 cm of the tumor) and distant uninvolved lung tissue. While keeping the samples constantly frozen, five 10-μm thick sections were cut, trimmed of excess OCT from around the tissue, and placed into frozen 1.5 mL microfuge tubes. Following the addition of 200 μl homogenization buffer (40 mM HEPES, 125 mM NaCl, 5 mM KCl, 05% Tween-20 at pH 7.5 plus HALT protease inhibitor cocktail (Pierce)), the samples were homogenized in the microfuge tubes on ice with rotary pestle for 30 seconds, until no tissue fragments were visible. The samples were then spun in a centrifuge at 21,000xg for 10 minutes and filtered through a 0.2 μm multi-well plate filter into a sterile multi-well plate. Five μl aliquots were taken for micro-BCA protein assay (Pierce) and the rest of the sample was stored frozen and sealed in 96 well plates at −70°C.

Proteomic Profiling

Sample total protein was adjusted to16 μg/mL in SB17T buffer (40 mM HEPES, 125 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 0.05% Tween-20 at pH 7.5) for proteomic profiling. Samples prepared in this manner were analyzed on the SomaLogic biomarker discovery assay using Agilent slide readout that measures 820 human proteins as described previously [5]. The proteins against which the SOMAmers included in this assay were selected are given in Table S1. Briefly, this assay uses SOMAmers to transform protein concentration into a corresponding DNA concentration through a series of steps involving affinity binding and capture of biotin onto streptavidin beads. The final DNA concentration is measured as relative fluorescence units (RFU) from the fluorescent SOMAmer hybridized to a complementary probe on an Agilent array.

Since each of the eight tumors in the study varies in stage and histology, we did not assume that the RFU measurements from the tissue resections were normally distributed. Therefore, we tested the significance of differential expression between the eight tumor tissues and the sixteen healthy surrounding tissues using a non-parametric test, specifically the Mann-Whitney rank sum test. We chose to combine the two healthy tissue groups because this study contains a small number of total samples.

A separate two-tailed Mann-Whitney test was performed for each of the 820 human proteins measured by the SOMAscan platform, so we corrected for multiple comparisons by applying a false discovery rate (FDR) correction [36] and enforced a q-value threshold of 0.05 for all analytes.

Due to the low number of samples and the non-parametric nature of the Mann-Whitney test, we identified many significant analytes that were only differentially expressed by a very small magnitude. Therefore, we sorted the set of significantly differentially expressed analytes by their log ratios between the tumor samples and the healthy samples. For this paper, we chose to highlight the 36 analytes with the highest log ratio within the set of significantly differentially expressed analytes.

SOMAmer staining method

5 μm frozen tissue sections were immediately placed onto a charged slide (Superfrost Plus), and the slide was then immersed in a fixative solution of 100% ethanol (P-Selectin & ESAM) or acetone (all other SOMAmers) for at least 30 minutes. OCT medium was removed from slides by a 2-minute rinse in deionized water, followed by a 2 to 5 minutes rinse in selection buffer SB-T (40 mM Na-HEPES, pH 7.5, 52 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 0.05% Tween-20). 200 mM SOMAmer solutions made up in SB-T supplemented with 1 mM dextran sulfate (DS) were applied for one hour to rinsed sections and then washed for 2 minutes with SB-T at 1–5°C.

Antibody staining method

5 μm tissue sections were immediately placed onto a charged slide (Superfrost Plus), and the slides were then immersed in a 1–5°C 100% acetone fixative solution for at least 30 minutes. The slides were removed from the chilled acetone bath and immediately placed into 1–5°C, 1% paraformaldehyde/PBS pH 7.4 fixative solution for 8 minutes. After fixation the remaining OCT embedding medium was removed from slides by two 30-second rinses in tap water followed by a 5-minute rinse in selection buffer SB-T, pH 7.4. The tissue sections were then blocked for 30 minutes with 10% goat serum in SB-T, pH 7.4/50 mM glycine. Rabbit and mouse primary antibodies were diluted into SB-T 7.4 buffer and applied to the pre-blocked slides for at least one hour. Primary antibodies were washed from the slides for 5 minutes with SB-T, pH 7.4 and then incubated with either Dy549-goat anti-rabbit or Dy549-goat anti-mouse fluorescent secondary antibodies in SB-T, pH 7.4/300 nM DAPI for 30 minutes. The working concentration of the secondary antibody was 3 μg/mL. The slides were finally rinsed for 5 minutes in SB-T, pH 7.4 at room temperature and cover slipped with Fluoromount-G supplemented with 15 mM n-propyl gallate antifade reagent. Details regarding the antibodies used are given in Table 5.
Nikon 80i widefield microscope method

Fluorescence images were acquired with a Nikon 80i upright microscope equipped with Digital Sight DS-Ri1 color camera, mercury lamp, and optical filters appropriate for 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) and Cy3 imaging. Data were collected and analyzed with Nikon NIS Elements AR 3.2 software.

Nikon A1R confocal microscope method

Fluorescence images were acquired with a Nikon A1R ECLIPSE Ti-E inverted microscope equipped with a 32-channel PMT spectral detector unit (SDU). The Nikon SDU was used to subtract the signal arising from autofluorescence. The confocal imaging data was processed offline using the ROI spectral unmixing feature provided in the Nikon NIS Elements AR 3.2 software.

Image processing method

ESAM expression was quantified and scored in single cells with CellProfiler and CellProfiler Analyst, respectively. The version number of CellProfiler software used for this analysis was r10997 and can be downloaded at www.cellprofiler.org [37]. Image analysis was performed using 10× images acquired on a Nikon 80i DS-Ri1 color camera at full frame 4076×6116 pixel dimensions. CellProfiler quantified the number of ESAM+ cells, defined as cells with DAPI+ nuclei and ESAM+ immunofluorescence, after a flat illumination field correction. The binary classification measurement rule was generated with a gentle boosting based supervised machine learning classifier module in CellProfiler Analysis r1123011246 [38].

Table 4. List of potential NSCLC biomarkers identified from serum and tissue samples.

| #  | Protein Name | NSCLC Serum | NSCLC Tissue |
|----|--------------|-------------|--------------|
| 1  | Activin A    | Up          |              |
| 2  | Adiponectin  | Down        |              |
| 3  | AMPM2        | Up          |              |
| 4  | BCA-1        | Up          | Up           |
| 5  | Biglycan     | Down        |              |
| 6  | BMP-1        | Down        |              |
| 7  | C1s          | Up          |              |
| 8  | C9           | Up          |              |
| 9  | Cadherin-1   | Up          | Up           |
| 10 | Calpain 1    | Up          |              |
| 11 | Carbonic anhydrase III | Down        |              |
| 12 | Caspase-3    | Up          |              |
| 13 | Catalase     | Up          | Down         |
| 14 | CD30 Ligand  | Up          |              |
| 15 | CD36         | Down        |              |
| 16 | CDK5/p35     | Up          |              |
| 17 | CK-MB        | Down        |              |
| 18 | Contactin-5  | Down        |              |
| 19 | CKCL16, soluble | Down        |              |
| 20 | Endostatin   | Up          | Down         |
| 21 | ERBB1        | Down        |              |
| 22 | ESAM         | Down        |              |
| 23 | FGF-17       | Up          |              |
| 24 | Fibronectin  | Up          |              |
| 25 | FYN          | Up          |              |
| 26 | HSP 90a      | Up          |              |
| 27 | HSP 90b      | Up          |              |
| 28 | IGFBP-2      | Up          | Up           |
| 29 | IGFBP-5      | Up          | Up           |
| 30 | IGFBP-7      | Up          | Up           |
| 31 | IL-15 Ra     | Up          |              |
| 32 | IL-17B       | Up          |              |
| 33 | IL-8         | Up          |              |
| 34 | IMB1         | Up          |              |
| 35 | Insulyisin   | Up          |              |
| 36 | Kallkrein 7  | Down        |              |
| 37 | KPCI         | Up          |              |
| 38 | LDH-H 1      | Up          |              |
| 39 | LGMN         | Up          |              |
| 40 | LRIG3        | Down        |              |
| 41 | MAPK13       | Up          | Up           |
| 42 | MEK1         | Up          |              |
| 43 | Midkine      | Up          |              |
| 44 | MIP-5        | Up          |              |
| 45 | MMP-12       | Up          | Up           |
| 46 | MMP-7        | Up          | Up           |
| 47 | MRC1         | Up          | Down         |
| 48 | NACA         | Up          |              |

Table 4. Cont.

| #  | Protein Name | NSCLC Serum | NSCLC Tissue |
|----|--------------|-------------|--------------|
| 49 | NAGK         | Up          | Up           |
| 50 | NAP-2        | Down        |              |
| 51 | PARC         | Up          |              |
| 52 | P-Selectin   | Down        |              |
| 53 | PTN          | Up          |              |
| 54 | Renin        | Up          |              |
| 55 | RGM-C        | Down        |              |
| 56 | SCF sR       | Down        |              |
| 57 | SLPI         | Down        |              |
| 58 | sL-Selectin  | Down        |              |
| 59 | sRAGE        | Down        |              |
| 60 | Thrombospondin-1 | Up        |              |
| 61 | Thrombospondin-2 | Up        |              |
| 62 | TnATPase     | Down        |              |
| 63 | Tryptase β-2 | Down        |              |
| 64 | Ubiquitin+1  | Up          |              |
| 65 | uPA          | Up          |              |
| 66 | URB          | Up          |              |
| 67 | VEGF         | Up          | Up           |
| 68 | vWF          | Down        |              |
| 69 | YES          | Up          | Up           |

Proteins identified in both studies are shown in boldface font. doi:10.1371/journal.pone.0035157.t004
Table 5. Antibodies used in for confirmation of analyte presence.

| Antibody Target | Primary antibody dilution | Vendor | Location | Host | Poly/Mono-clonal |
|-----------------|---------------------------|--------|----------|------|------------------|
| TSP-2           | 1:1000 (0.35 μg/ml)       | Novus Biologicals | Littleton, CO | rabbit | poly |
| TSP-2           | 1:500                    | Anti-TSP-2 clone 67, a gift from Dr. Paul Bornstein | Seattle, WA | rabbit | poly |
| MMR/CD206       | 1:500 (0.5 μg/ml)         | R&D systems | Minneapolis, MN | rat | mono |
| ESAM            | 1:250 (1 μg/ml)           | R&D systems | Minneapolis, MN | mouse | mono |

Supporting Information

Table S1 SomaLogic Selection Targets. The proteins against which each of the SOMAmers used in the SOMAscan assay were selected is listed.

Acknowledgments

We thank all of our Soma Logic colleagues for the years of work that went into developing the tools for this technology. The authors would like to thank Dr. Paul Bornstein for providing Thrombospondin-2 antibodies.

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