Identification of Serum Biomarkers for Lung Cancer Using Multiplexed Immunoassays

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Abstract

Background: Symptoms of lung cancer (LC) often do not appear until the disease is advanced; only 15% of LC cases are discovered while the tumor is in the early stages of development. Carcinogen exposure, asthma and smoking have been determined to be risk factors for the development of LC. Early detection of LC will likely have a major impact on the natural history of the disease and facilitate curative treatment. The objective of this study was to apply multiplexed immunoassays to identify a panel of biomarkers for early detection of LC.

Methods: Normal (NO) serum controls (n = 30) from healthy volunteers and lung cancer patients (n = 30) were acquired from a commercial vendor. Baseline (pre-treatment) serum specimens from individuals with asthma (AST; n = 28) and lung cancer risk (LCR; n = 73) were available from clinical trials of two novel agents that are being developed by JBNI Inc. for the respective indications. Serum levels of 59 cytokines, growth factors and biologically active peptides were quantified using multiplexed immunoassays using the Luminex platform to identify biomarkers that are expressed in a significantly different manner in individuals with LC, LCR, or AST in comparison with NO subjects. Data were reduced using nearest neighbor cluster analysis with squared Euclidian distance to separate patients into groups across analytes with inter-pathology comparisons determined using Student's t test.

Results: Multiple analytes showed highly significant differences ($p < 0.0002$) between LC and healthy controls as single indices of pathologic state. In addition we were able to differentiate AST from LC ($p < 0.002$) and LCR and LC ($p < 0.0001$) using a panel of thirteen markers in various combinations. Using multiplexed assays we found significant differences in biomarker levels in sera of LC compared to NO, in NO compared to AST and LC compared to AST samples. Our results support an extended multiplexed immunoassay-based analysis of serum biomarker profiles as supplementary tools for the diagnosis of pathologic and as an aid in the development of novel agents for prevention, early detection and treatment of LC.

Conclusions: We have identified a group of markers having high inter-pathology discrimination power that are capable of reliably differentiating AST and LC from control specimens. This panel remains to be validated in a larger set of specimens but we are confident that these measures will produce clinical assays capable of reliably diagnosing lung pathology.

Support: JBNI Corporation

Materials and Methods

NO serum controls (n = 30) from healthy volunteers and from LC patients (n = 30) were acquired from a commercial vendor. Baseline (pre-treatment) serum specimens from individuals with AST (n = 28) and from LCR (n = 73) were acquired as part of a clinical trial of two novel agents for the treatment of asthma.

Serum levels of 59 cytokines, growth factors and biologically active peptides were quantified using multiplexed immunoassays using the Luminex platform to identify biomarkers that are expressed in a significantly different manner in individuals with LC, LCR, or AST in comparison with NO subjects.

Samples were collected by venipuncture into sodium citrate Vacutainer tubes. Plasma was separated by centrifugation, the supernatant was aliquoted into 96-well plates and frozen at -80 °C.

For multiplex immunoassay the samples were thawed on ice and specimens were prepared according to the instructions of the individual manufacturer kit preparation protocols. Various kits were used to assemble the panels of assayed targets. Kits were sourced from Linco Research Inc. (St Charles, MO) and R&D Systems Inc. (Minneapolis, MN). The samples were assayed using a Luminex 100 (Luminex Co., Austin TX).

Statistical analyses were accomplished by first breaking out data for each pathology for each marker measured into subgroups by univariate random seed k nearest neighbor cluster analysis. The subgroups identified were then compared across pathologies within a given marker using Student's t test. Markers for further analysis were selected by collecting those markers having the most significant and consistent matrices of inter-pathology t values.

The selected markers were extracted and grouped into combinatorial panels and subjected to further multivariate cluster analysis across pathologies followed by inter-subgroup t testing. Those markers found to present optimum combinations of characteristics were extracted, grouped together and again broken into clusters.

Comparative analyses of inter-pathology differences were accomplished by performing cluster analyses for identified sets of markers. Individual specimens contained within the generated subgroups were counted according to pathology by sub-group and the counts were used to calculate the discriminative capability of any given panel of markers.

Results

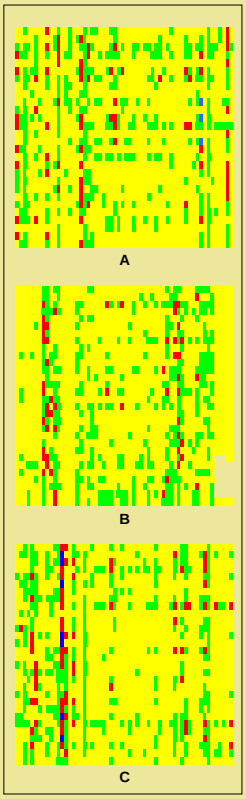

Figure 1. Univariate cluster patterns for 59 markers in lung cancer (A), normal (B) and asthma (C). Data are rank ordered by color with yellow, green, red and blue representing progression from low to high intensities respectively.

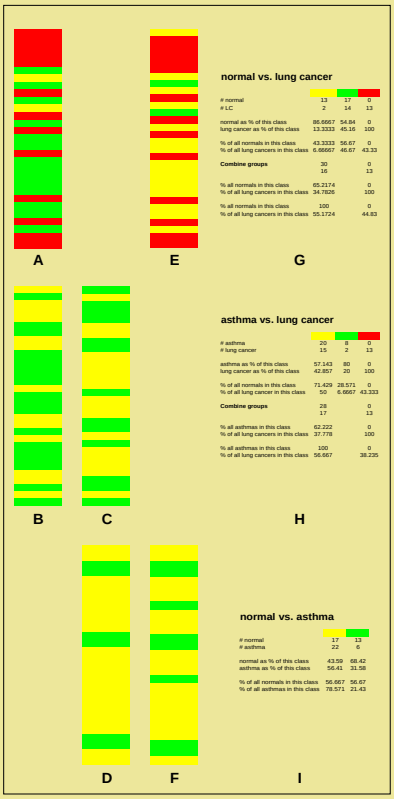

Figure 2. Multivariate cluster analysis for 12 selected markers: IL-1ra, EGF, IL-6, IL-12, IL-17, IL-1α, TNFα, MCP-1, MIP-1β, IP-10, and VEGF, having relatively poor inter-pathology differentiating power showing rank stratification of patient samples between lung cancer (A, E) , normal (B, C) and asthma (D, F) patient groups. Statistical summary for inter-group comparisons for normal vs. lung cancer (G), asthma vs. lung cancer (H) and normal vs. asthma (I) .

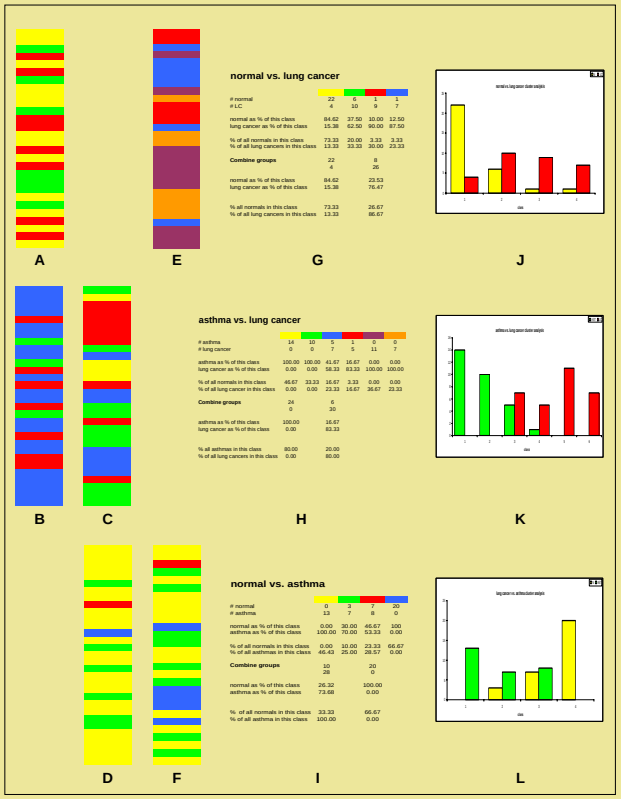

Figure 3. Multivariate cluster analysis for 13 selected markers* showing rank stratification of patient samples between lung cancer (A, E) , normal (B, C) and asthma groups (D, F). Statistical summary for inter-group comparisons for normal vs. lung cancer (G), asthma vs. lung cancer (H) and normal vs. asthma (I). Histogram plots of abundance vs. class rank for normal vs. lung cancer (J), asthma vs. lung cancer (K) and normal vs. asthma (K).

* Patent pending

Summary

Given the wide intra- and inter-individual variance within a pathology for many markers, we adopted a multi-marker approach to pathology classification characterizing disease status using a fingerprint of molecular signatures designed to compensate for the wide variances encountered in single marker measurements.

We have identified several panels of markers that stratify and correctly classify the particular pathology present in the patient. The minimum predictive percentage observed was 76% for the comparison of LC to N specimens across 4 selected markers. We identified several combinations of markers capable of clearly differentiating N from LC with 86% probability and that further partition LC patients into at least 3 subgroups, with different sets of markers statistically driving the formation of each of the subgroups. The correlation of these sub-groups with patient characteristics such as sex, age *etc.* and disease status such as stage, treatment status, *etc.* is ongoing.

In AST specimens we were able to use the same sets of markers identified as predictors for LC status resulting in the speciation of four subgroups, one of which contained 100% asthma patients, one that contained 100% normal patients, and two which contained various ratios of normal and asthmatic patients. Quantification of these markers allowed us to reliably differentiate AST from LC with >80% probability.

During the course of these analyses we discovered that the LCR group, which is composed of samples from smokers, was diverse in composition to the point that inclusion of these specimens in the comparative analyses yielded no meaningful results (data not shown) and thus these specimens were excluded from further analysis.

These results will be confirmed and validated in studies to be conducted on much larger sample sets in the near future, which may allow us the statistical power to discriminate sub-pathologies and/or time/development stage of the specimens from diseased patients.