Introduction
Cancer is a leading cause of death worldwide, with 8.2 million deaths attributed to this disease in 2012. The most common causes of death by cancer are lung, liver, stomach, colorectal, and breast. Cancer rates are increasing as the world's population ages; and the worldwide cost of cancer is estimated at 1.16 trillion US dollars per year. However, many cancers have a higher probability of cure if detected early and treated properly. Early treatment requires early diagnosis, which can be enabled by screening of asymptomatic populations with high risk factors. Such screening requires tests that are sensitive and specific, as well as amenable to high throughput.

Cancer Autoantibodies
While the immune system is an efficient protector of the human body from foreign pathogens, it is now well understood that it also plays a role in the prompt identification of endogenous anomalies such as those that happen in the earliest stages of tumorigenesis. While tumor cells produce unique neo-antigens, they also aberrantly express self-antigens. The antibodies to self-antigens may be the result of over-expression or altered tissue-specific expression, mutation that results in new antigenic epitopes, uncontrolled posttranslational modification such as abnormal glycosylation, or unregulated degradation. The products of such processes, referred to as tumor-associated antigens (TAAs), cause a loss of self-tolerance similar to that which occurs in autoimmune diseases. This abnormal immune response is expressed as specific autoantibodies.

A great deal of research effort has focused on harnessing cancer-associated autoantibodies as early indicators of cancer, based on the nature of the autoimmune response. The initial immune response to TAAs is a local one, initiated by a very small number of tumor cells at an otherwise undetectable early stage. However, a systemic B cell response then provides amplification of TAA-specific autoantibodies that results in high titers in the blood stream during pre-symptomatic and pre-diagnostic stages of disease. These autoantibodies can be readily measured in the sera of cancer patients, and their inherent stability and long half-life enables assay reproducibility. Expression of these autoantibodies also changes with disease states, making them useful as potential tools for monitoring and prognosis. Multiple efforts are underway to use these autoantibody levels for early cancer detection, prediction of recurrence, metastasis, and response to therapy.

Multiplex Assays are Essential
Biomarker researchers and those using autoantibodies to detect cancer recognize the necessity of using panels of analytes, rather than single biomarkers, to develop clinical tests with the necessary sensitivity and selectivity. In fact, single autoantibody biomarkers for cancer have not been accepted into clinical practice for cancer detection. The panel approach may offer improved sensitivity and preserve a high level of specificity. It also provides a greater probability that any patient will reveal a response to at least one of the antigens. Such panels may also distinguish among tumor types, stages of disease, and response to treatment.

Luminex xMAP® bead-based and highly-multiplexed assays are ideal for this application. In fact, "the development of bead-based immunoassay platforms has had a significant impact on the field of serum biomarker discovery and development." They add improved kinetics and flexibility to the known advantages of ELISA. Perhaps the biggest advantages of bead-based systems is a high capacity for multiplexing that enables reduced sample and reagent volume, high throughput and automation capabilities, and the generation of a large amount of information in a single experiment. In addition, Luminex bead-based assays are "reliable, accurate, cost-effective, highly sensitive, and have rapid turnaround time for results".

Luminex xMAP Technology
Luminex internally dyes bead sets with precise concentrations of fluorescent dyes, resulting in 500 distinctly colored bead sets (Figure 1). Each bead set can be coupled with reagents for specific bioassays such as antigens, antibodies, cytokines, or oligonucleotides. Any combination of bead sets can be used in a single assay, enabling multiplexing of up to 500 analytes from a single reaction volume. The bead mixture is incubated with the sample and detected on a Luminex instrument using a reporter dye to quantify the amount of bound analyte.
Depending upon the instrument used, up to 500 bead types can be used in each well of a 96- or 384-well plate, thus generating a high-throughput assessment of a large number of protein or oligonucleotide targets. This microsphere based “liquid array” system for measuring analytes is unique in its ability to provide both high-throughput and high-content data, and researchers are able to easily scale the number of analytes measured as well as customize both the assays and types of applications.

Lung Cancer

Diagnosis of 75% of non-small cell lung cancer (NSCLC) occurs at an advanced stage not amenable to surgery, and it has an overall 5-year survival rate of <15%. If diagnosis occurs at stage I however, the 5-year survival rate is over 50%. Thus, an autoantibody serum assay that could unambiguously identify patients with NSCLC at a very early stage could provide an ideal complement to current computed tomography (CT)-based screening protocols. Spiral CT-based screening is limited by a high rate of false positives that lead to unnecessary surgery and the need for multiple measurements to improve specificity.

A study to identify autoantibody biomarkers in ten patients with lung adenocarcinoma yielded 16 autoantibodies expressed in NSCLC serum versus controls. These were combined with autoantigens previously shown to have potential diagnostic value to develop a Luminex xMAP assay. This assay was used to evaluate a second patient cohort of 196 patients that included 117 NSCLC subjects. Following multivariate statistical analysis of the data, a six-autoantibody algorithm for detecting NSCLC among high-risk patients was developed.

Although p53 antibodies have been shown to be associated with NSCLC, this biomarker was not useful as part of the six-analyte panel. This panel consisted of autoantibodies to inosine-5′-monophosphate dehydrogenase (IMPDH), phosphoglycerate mutase, ubiquilin, annexin I, annexin II and heat shock protein 70-9B (HSP70-9B). Performance of this panel was encouraging against the 196 patient second cohort, with only 13 patients misclassified overall. The result of the study indicated that the algorithm could be well suited to help determine which patients required further diagnostic evaluation.

A follow-up study starting with the same 6-analyte panel was conducted to determine if autoantibody screening could determine whether lymph node metastasis had occurred. Many NSCLC patients present with regional (lymph) or distant metastases, with the five-year survival rate being only 24% or 4%, respectively. This second study involved the identification of 11 additional autoantigens in sera from 20 NSCLC patients that were associated with progression to the locoregional lymph nodes. These and 14 autoantigens known to be relevant to early disease detection were used to develop a Luminex xMAP assay that was used to screen 107 NSCLC patients for autoantibodies against the autoantigens. Multivariate statistical analysis of the results identified an optimal combination of biomarkers when used along with the original 6-analyte panel.

The new panel consisted of autoantibodies against the six following TAA s: ubiquilin-1, hydroxysteroid (17β) dehydrogenase variant I (HADH) and triosephosphate isomerase (TPI) (identified in the second study), as well as tumor necrosis factor alpha (TNF-α), TNF-RI (receptor I) and macrophage inflammatory protein one (MIP-1) (retained from the original panel). This new algorithm improved the classification efficiency of NSCLC to 96% for the revised panel. The false-positive rate improved from 11.2% to 2%. This new panel was used to re-analyze 15 cases misclassified by CT-based protocols. Of these, the panel correctly re-classified seven as node positive and four as node negative. Two of the four remaining cases were misclassified by both. This second study concluded that a simple, cost-effective blood test for evaluation of metastatic progression may be very complementary to radiographic staging, and it may help improve the overall accuracy rate.

A third NSCLC study by the same research group was conducted to determine if the Luminex xMAP autoantibody panels could be used to predict recurrence in stage I, with the end goal of improving survival. A total of 43 autoantibody biomarkers were evaluated against 79 patients with resectable NSCLC, in three groups: stage I without recurrence; stage I with recurrence, and node-negative. All patients underwent anatomic resection, and peripheral blood was collected before surgery. A prognostic classification autoantibody panel was then evaluated using multivariate statistical methods. A total of 28 biomarkers was significant for recurrence, of which 10 were strongly prognostic. A 6-analyte algorithm was then generated that was 77% accurate for predicting recurrence in stage I patients, with a sensitivity of 74% and a specificity of 79%. These results hold promise that further improvement of this panel could enable aggressive recurrence monitoring to improve survival, or stratify patients for adjuvant therapy.

Another autoantibody xMAP panel has been developed for detection of lung cancer by a Chinese research team. This approach used the HaloTag® technology (Promega) to generate recombinant antigen proteins that could be directly bound to linkers on the beads recognized by the tag, without prior purification, thus streamlining the bead preparation process. The panel used consisted of seven antigens: p53, NY-ESO-1, livin, ubiquilin I, Baculoviral IAP repeat-containing protein (BIRC), nucleopore α, and peroxiredoxin (PRDX). All seven were found to be present at much higher levels in lung cancer patients, relative to healthy controls, and six of these are shown in Figure 2. A multivariate statistical model was then developed that provided over 80% accuracy in detecting lung cancer versus healthy controls.
Ovarian Cancer

Ovarian cancer is the most lethal gynecologic cancer, even though aggressive surgery and chemotherapy treatments are in use. Ovarian cancer is a group of diseases and tumor types with distinct pathogenesis and morphologic features. It can be classified into two broad categories (type I and type II) based on their distinct pathogenesis. Type II tumors are highly aggressive and often present in advanced stage at diagnosis, when current available therapies are least effective. These tumors account for 90% of ovarian cancer deaths. A sensitive and specific screening test for type II tumors that can detect them before the disease manifests itself, and before metastasis occurs, could help reduce this death rate.

A study has been conducted to develop such a screening test using a MILLIPLEX® MAP Cancer Biomarker Panel kit based on xMAP technology. The kit was specific for six of the 14 biomarkers that were evaluated, including migration inhibitory factor (MIF), leptin, prolactin, cancer antigen 125 (CA-125) and insulin growth factor II (IGF-II). The other biomarkers were measured using ELISA. Five of the autoantibodies measured using xMAP technology had significantly different plasma levels between healthy controls and Type II ovarian cancer patients when examined individually, as did two autoantibodies measured using ELISA. CA-125 provided the greatest discriminatory power, followed by IGF-II. When p53 autoantibody levels were combined with CA-125 levels, the sensitivity was the highest for discriminating type II cancers, at 85.7%.

Other Cancers

The lack of specificity and sensitivity of the prostate serum antigen (PSA) test for prostate cancer is widely recognized, and autoantibody panels have the potential to increase both. An autoantibody panel consisting of epitopes from cancer antigens NY-ESO-1, XAGE-1b, synovial sarcoma, X breakpoint 2 and 4 (SSX-2,4), alpha-methylacyl-CoA racemase (AMACR), p90 autoantigen and lens epithelium-derived growth factor (LEDGF), plus antibody to PSA, was constructed using xMAP technology. This novel approach thus enabled measurement of prostate cancer autoantibodies and PSA levels in the same xMAP assay.

An index value was calculated for each autoantibody, as the ratio of the mean fluorescent intensity (MFI) generated by the biomarker peptide, versus the MFI of a β-galactosidase control peptide. Prostate cancer patients exhibited a much broader range of index values for each autoantibody than patients with non-malignant conditions (Figure 3).
The differences between the two groups were highly statistically significant, with the exception of AMACR autoantibodies. A combined autoantibody (A) plus PSA index was created to determine the probability of prostate cancer. Adding any single autoantibody had little effect on the sensitivity of the PSA test. However, the addition of all 6 autoantibodies (A+PSA) significantly increased the sensitivity, specificity and accuracy of prediction of the PSA assay (Table 1). This study thus identified potential benefits for the A + PSA assay in differentiating prostate cancer from non-malignant conditions.

Table 1. Comparison of A+PSA index and PSA based on mean values at three different dilutions.

<table>
<thead>
<tr>
<th>Variables</th>
<th>PSA alone in all patients</th>
<th>A+PSA in all patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>52% (68/131)</td>
<td>79% (103/131)</td>
</tr>
<tr>
<td>Specificity</td>
<td>79% (95/121)</td>
<td>84% (102/121)</td>
</tr>
<tr>
<td>False positive</td>
<td>21% (26/121)</td>
<td>16% (19/121)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>65%</td>
<td>81%</td>
</tr>
<tr>
<td>AUC</td>
<td>0.66</td>
<td>0.91 P &lt; 0.0001</td>
</tr>
</tbody>
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One of the most fatal cancers diagnosed in the United States, pancreatic cancer, develops slowly over many years. However, most patients present with late stage disease that is resistant to treatment. An early diagnostic tool would thus have the potential to greatly increase probability of survival. A recent study was conducted to test the utility of an autoantibody panel for detection of pancreatic cancer. Three antigens identified as potentially promising biomarkers in exploratory studies were included in the xMAP-based assay panel: CTDSP1 (carboxy-terminal domain, RNA polymerase II, polypeptide A, small phosphatase 1), MAPK9 (mitogen-activated protein kinase 9), and NR2E3 (nuclear receptor subfamily 2, group E, member 3). The panel was used to test sera from 300 cases and 300 controls from a population-based case-control pancreatic cancer study. The cases had higher levels of all three autoantibodies than did the controls, indicating that the panel has potential to be useful in the diagnostic screening and prognosis of pancreatic cancer.

Luminex xMAP Technology

Luminex provides the only flexible and open multiplexing technology that is used by several market leaders to provide assays for both gene and protein expression. Unlike conventional technologies that can only measure one or a few biomarkers, researchers have the capability to easily scale up or down the number of biomarkers measured and to customize assays. xMAP technology combines advanced fluidics, optics, and digital signal processing with proprietary microsphere technology. Featuring a flexible, open-architecture design, xMAP technology can be configured to perform a wide variety of bioassays quickly, cost-effectively and accurately. Focused, flexible multiplexing of 1 to 500 analytes meets the needs of a wide variety of applications, including genotyping, protein expression profiling, gene expression profiling and HLA testing for infectious disease, autoimmune disease, and genetic disease research.

All of the microsphere bead assays described in this white paper were developed using xMAP technology to provide unique multiplexed assays for autoantibodies that could be used for cancer screening, diagnosis, and prognosis. The open architecture of the system made it feasible for the researchers to create their own assays, or obtain commercially available kits. An ever-expanding menu of assays for other applications is also available from Luminex and its commercial partners.

Partial List of Institutions using Luminex xMAP Technology for Autoantibody Research

- University of Pittsburgh Cancer Institute, Hillman Cancer Center, Pittsburgh, PA, USA
- Program in Molecular Biology and Genetics, Karmanos Cancer Institute and Department of Pathology, Wayne State University School of Medicine, Warren, Detroit, MI, USA
- Instituto de Estudios de Ciencias de la Salud de Castilla y León’s Instituto de Investigación (IESCYL), Spain
- Dana-Farber Cancer Institute, Harvard Medical School, Boston MA, USA
- Rush University Medical Center, Chicago, IL, USA
- Division of Pulmonary and Critical Care Medicine, University of Kentucky, Lexington, KY, USA
- Center for Molecular Medicine, Zhejiang Academy of Medical Sciences, Hangzhou, Zhejiang, Peoples Republic of China
- Johns Hopkins University School of Medicine, Baltimore, MD, USA
- Department of Urology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA
- Department of Epidemiology and Biostatistics, University of California San Francisco, San Francisco, CA, USA
REFERENCES