Liquid ImmunoBiopsy™ – an accurate, fast, and broad platform to non-invasively measure cellular immune responses as a diagnostic method for early disease states

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This article describes a novel cellular immune response diagnosis platform, its importance as an in vitro cellular response assay, and summarizes clinical study results deploying this platform for the detection of early stage of Lung cancer.

Introduction

There is great importance to an assay that can monitor in vitro cellular immune responses (primarily T and B cells) to antigenic stimuli, within a few hours, to determine immune activation levels in response to a specific stimulus. To date there are no other laboratory tests that provide a quick and accurate answer. Such an assay can help diagnose early stages of cancer by using tumor-associated peptides, provide cellular immune status of vaccinated people to SARS-COV-2 by using virus spike peptides, and measure other cellular immune statuses to diseases such as allergy, autoimmune, immunodeficiency, antimicrobial immunity, or to follow up the effect of treatment. In general, an effective in vitro response of the cells to re-stimulation with a specific stimulant indicates that the immune cells were previously exposed to the specific stimulant. Importantly, it indicates that the cells are able to produce an immunological response to it.

There are several classical methods to test lymphocytes' function. Mixed leukocyte culture (MLC) determines histocompatibility by co-culturing peripheral blood mononuclear cells (PBMC) of a potential donor with those of an allograft recipient. MLC takes 7-8 days to get results and involves the use of 3H-thymidine radiolabeling. Limiting dilution assay (LDA) also assesses histocompatibility between two parties. It determines the precursor frequencies of cytotoxic and helper T lymphocytes. The duration of this test is generally longer than MLC and takes 7–18 days. Lymphocyte transformation test (LTT), in contrast to MLC and LDA, measures lymphocyte responses toward nonspecific stimuli (mitogens) or specific stimuli (antigen). A proliferative response shows that antigens of the respective microorganism are presented by antigen presenting cells, and are recognized by pre-existing, antigen-specific T lymphocytes. The duration of this test is 8-10 days. A more recent method to test the

function of lymphocytes is the enzyme-linked immunospot (ELISpot) assay. It is a sensitive and quantitative method to detect cytokine production level in cell culture supernatant, and after growing cells with stimulant antigen. The duration of this test including cell culturing is 2-12 days. Various flow cytometry assays that measure lymphocyte functionality include tests that are based on the detection of cell divisions by fluorescent CFSE staining, use of multimer staining of HLA restricted peptides with their T cell receptor, use of other staining of cells receptors, or measurement of proteins that correlate with cells activation. Like ELISpot, these types of tests need to cell culture for 2-12 days. ImmuKnow test measures the response of $CD4^+$ T-helper lymphocytes to the mitogen phytohaemagglutinin-L (PHA), a general stimulator. It measures the amount of ATP produced by the cells following nonspecific stimulation. The duration of this test is two days (1,2). While the methods described are non-invasive or devoid of the radiation risk of imaging, they all require days of execution, are cumbersome to perform, and there are no uniform standards (positive and negative controls, units) in performing these methods by different users.

Savicell Diagnostics Ltd.'s Liquid ImmunoBiopsy™ platform is based on measurements of metabolic activity profiles of immune cells. Studies show that activation of immune cells requires changes in the way metabolic energy (ATP molecules) is generated. Immune system cells alter their energy generation in order to obtain an effector function. Usually the shift is from the oxidative phosphorylation cycle into an aerobic glycolysis cycle. This shift provides immediate energy that gives the immune system the ability to attack the foreign antigen (3, 4, 5). Peripheral blood mononuclear cells (PBMCs) are Immune cells that are isolated from peripheral blood. PBMCs are mixed with disease associated antigens. Stimulation of PBMCs by these antigens takes place only when these PBMCs were previously exposed to these antigens in the body. This in vitro stimulation induces a rapid shift into aerobic glycolysis metabolic pathway, as a necessary step for PBMCs activation (6). The shift into glycolysis is measured by following acid production, a final product of the oxidative phosphorylation and glycolysis cycles, that is secreted from the cells into the reaction medium. The Liquid ImmunoBiopsy™ platform measures the acidification rate in the extracellular environment of PBMCs, in varying conditions. It then constructs a metabolic profile as an accurate indicator of PBMCs' reactivity toward disease agents. The test duration from receiving the blood sample to result is only five hours.

Using the Liquid ImmunoBiopsy™ test to detect early-stage lung cancer

Lung cancer remains the leading cause of death from cancer, worldwide. Unfortunately, approximately 75% of patients are diagnosed at an advanced stage of the disease (III/IV) (7) and despite recent advancements in treatment, survival remains poor. Obviously, developing early detection diagnostic methods, especially non-invasive methods, is a critical component to raising the overall survival rate and prognosis for lung cancer (8).

Materials and methods

Metabolic Activity (MA) assay: A description of the assay can be found in a previously published article (9). In brief, immune cells PBMCs are isolated from blood samples using Lymphoprep™ kit (Axis-Shield). A panel of 15 lung cancer specific stimulants, a general stimulant as positive control (the

mitogen phytohaemagglutinin-L -PHA), and assay reaction medium as negative control, were loaded into a black, non-binding, multi-well plate. Each stimulant or control is loaded in triplicate. An equal volume of PBMCs from $5 \cdot 10^6$ cells/ml solution is added to each plate well together with a fluorescent probe 8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS) which is used to monitor pH changes. The multi-well plate is inserted into a plate reader device that documents the fluorescent signal received from each plate's well in continuous reading for 3 hours. The raw data from the plate reader device are processed instantly using a custom, and proprietary Savicell software.

Demographics

Subjects were enrolled between 03-24-2019 and 03-09-2021 in three medical centers. In all cases, the study received Helsinki approval, and subjects read and signed a dedicated consent form. The same inclusion and exclusion criteria were applied as described in our previous published article (9). The reference standard for lung cancer is biopsy or surgery.

Control subjects were age and sex matched with lung cancer subjects. Subjects with different types of lung cancer and different stages of lung cancer were included, with emphasis on early stages.

Table 1: Demographics and clinical characteristics for participating subjects (n=245). [a] The age of subjects at blood withdrawal; [b] Subjects with at least one pack-year in their history, who have not smoked in the past 30 days.

Characteristic		**Lung cancer group (n=111)**	**Control group (n=134)**	**All (n=245)**
Age (years)[a]		67.9 ± 8.8	52.4 ± 13.5	59.4 ± 13.9
Sex	Male	74	73	147
	Female	37	61	98
Smokers	Current	61	47	108
	Former[b]	30	18	48
Clinical stage	I	39	-	-
	II	21	-	-
	III	22	-	-
	IV	24	-	-
	N/A	5		
Histological type	Adenocarcinoma	77	-	-
	Squamous cell carcinoma	21	-	-
	Other	13	-	-

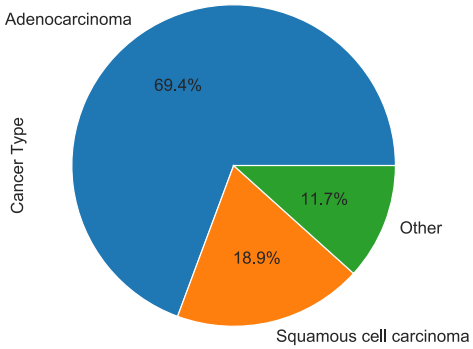


Figure 1: Non-small cell lung cancer (NSCLC) type distribution (n=245).

Data analysis

Each subject was assigned a datasheet containing fluorescent optical density values of plate wells as a function of time. The fluorescent readings were transformed into values which correspond to the acidity of the sample. We modeled the biological progression of the immunological response and extracted a set of optimal features to use in our assay.

Machine Learning (ML) models were trained on the extracted features using logistic regression implemented by the publicly available scikit-learn Python library. Models were evaluated using 5-fold stratified cross-validation (CV). Repeated evaluations with different random, cross validation train/test splits were performed to verify result stability.

Results

Results from a cohort of 245 subjects that processed in the period Mar. 2019 to Mar. 2021 are presented in Table 2 and Figure 2.

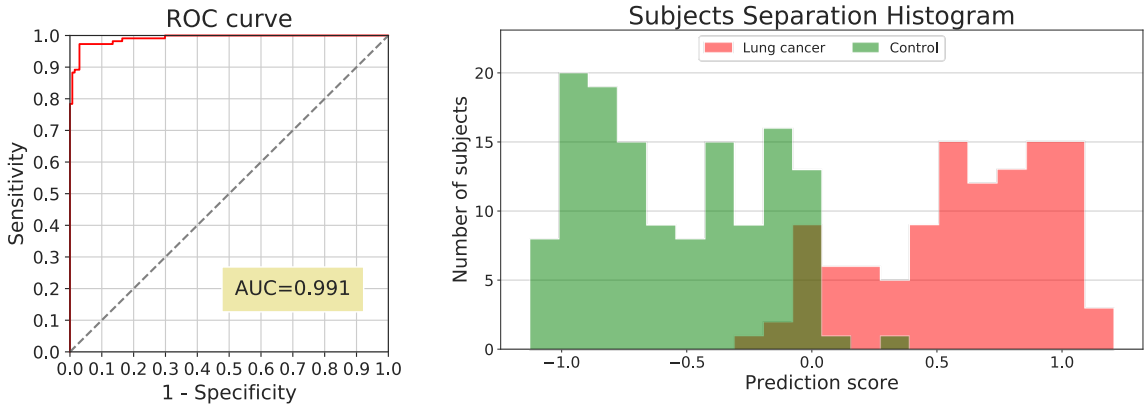
Table 2: Performance was evaluated using a stratified 5-fold cross validation. Confidence interval (CI) was calculated using Wilson score interval. Clinical stages I-II and III-IV were combined for sensitivity calculation; N/A indicates cases where the cancer clinical stage was not available. The Area Under the Curve (AUC) is a measure of separation between lung cancer and control subjects.

Performance measures		**CI (95%)**
Specificity	97.0%	(93% - 99%)
Sensitivity	97.3%	(92% - 99%)

Sensitivity per stage

Stage	**# of Donors**	**Sensitivity**
I	39	97.4%
II	21	95.2%
III	22	100.0%
IV	24	95.8%
N/A	5	100.0%

Positive predictive value (PPV)	96.4%
Negative predictive value (NPV)	97.7%
Area under the ROC curve (AUC)	0.991



(a) (b)

Figure 2: receiver operating characteristic (ROC) curve (a) and histogram of prediction scores (b), which visualizes the separation between lung cancer and control subjects.

Discussion

This study results indicate that the Liquid ImmunoBiopsy™ platform identifies early-stage lung cancer with high sensitivity and high specificity within 5 hours. The increased sensitivity and specificity of the recently tested cohort (n=245) versus previously shown (9) is the result of fine tuning the assay protocol in order to calibrate concentrations of stimulants and cells, select the most suitable stimulants, qualify quality control methods, and more. In addition, specificity was further enhanced by the detection of specific antibodies to lung cancer specific stimulants in subjects' plasma using our proprietary ELISA (Enzyme-Linked Immunosorbent Assay) protocol. This combined humoral and cell immune test provides enhanced specificity in the lung cancer clinical study, increasing specificity by about 3% to 97%.

The sensitivity and specificity obtained by Savicell's Liquid ImmunoBiopsy is much higher than the results reported in the literature (11,12) for stages 1 & 2 of lung cancer. Early detection of lung cancer saves life. The accuracy superiority for early stages by ImmunoBiopsy can be explained by the fact that immune cells, in lung associated lymph nodes, reach the malignant cells in the lung when the tumor is young, small and has yet to develop its ability to evade immune recognition. This later evasion by the immune cells can be the result of the failure of the immune system to be adequately activated, by the induction of immune tolerance, or by other inhibitory mechanisms that allow tumors to escape immune detection and elimination (13). Recognition of lung cancer antigens (stimulants) by immune cells is possible and results with immediate shift to the glycolysis pathway, enabling an effective local immunity. Tumor-exposed immune cells (in very early stage) reach peripheral blood, and repeated in

vitro exposure will result in a shift to the glycolysis metabolic pathway. This shift is detectable by Savicell's metabolic profile test.

Other tests are focused on detecting circulating biomarkers Including: tumor DNA, tumor antigens, tumor cells, exosomes, extracellular vesicles. These biomarkers are released to peripheral blood primarily when the tumor reaches a certain size in a later stage of the cancer.

We have previously shown (9) that COPD or smoking habits did not affect the test results, which supports other findings that diseases produce specific signatures in the metabolic profiles that can help distinguish between various ailments such as cancers, autoimmune diseases, and infectious diseases (10).

Current diagnostic methods (i.e., CT, PET, LDCT, radiography) have high sensitivity but low specificity. False positive rates of 96.4% for LDCT and 94% for radiography (14) lead to a large number of unnecessary follow-up procedures. These procedures are expensive, invasive and carry significant complication risks. There is an important need for the Liquid ImmunoBiopsy™ test with its high specificity and sensitivity that can assist in the diagnosis of indeterminate nodules and significantly reduce the number of false positive results. In addition, there is still a major unmet need for a safer, cost-effective liquid biopsy test that can help screen for lung cancer in the broader past and current smoker population.

The broad potential of this immunometabolism-based platform may also allow it to extend to other types of diseases, as well as to treatment monitoring and therapy selection.

In conclusion, the Liquid ImmunoBiopsy™ platform is a new, promising, noninvasive platform that measures the metabolic state of the immune system as a direct indicator of cellular immune responses (primarily T and B cells) to antigenic stimuli. It can act, *inter alia*, as a diagnostic method to detect early stages of lung cancer, with low material costs and quick results. Furthermore, the combination of low dose chest CT scans with the Liquid ImmunoBiopsy platform may reduce the need for follow-up of suspected lung nodules, preventing unnecessary radiation exposure and may also decrease the number of unnecessary invasive procedures with their associated complications. In addition, the ImmunoBiopsy™ test for lung cancer can help improve adherence to routine medical screenings in high risk populations through the use of a patient-friendly blood test. A larger prospective clinical validation is next step.

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