Quantitative multiplex analysis of immune checkpoint protein expression in circulation and in the tumor microenvironment

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An Important Update:
MILLIPLEX® MAP Human Immuno-Oncology Checkpoint Protein Panel (Cat. No. HCKPMAG-11K) is now available as a 16-plex multiplex kit. It includes the 15 checkpoint protein biomarkers reported in this 2017 AACR poster and an additional immune regulator biomarker, TLR-2. You can find more information on this 16-plex panel at: www.emdmillipore.com/milliplex.

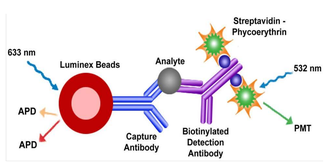
Abstract

Immune checkpoint inhibitors have been proven to be an effective method in improving antitumor immune response. Many immune checkpoint proteins are expressed as soluble forms in circulation and in the tumor and tumor microenvironment. Here we report the development of bead-based Luminex® multiplex assays for the quantitative profiling of co-inhibitory and co-stimulating immune checkpoint proteins CTLA-4, PD-1, TIM-3, LAG-3, HVEM, GITRL, BTLA, CD27, CD28, CD40, GITR, PD-L1, CD80/B7-1, CD86/B7-2, and ICOS (Cat. No. HCKPMAG-11K, which also includes an anti-tumor immune regulator, TLR-2*). In order to explore the use of soluble immune checkpoint proteins as putative cancer biomarkers, we used these multiplex assays to measure checkpoint protein levels in serum samples from breast cancer patients, colon cancer patients, and a corresponding set of normal serum samples. Analysis of the soluble checkpoint protein signatures generated from this multiplex approach revealed a significantly elevated level of soluble TIM-3 protein in the breast cancer serum samples and in the colon cancer serum samples compared to the healthy serum controls ($p<0.001$). In addition, we analyzed the immune checkpoint protein expression profiles in lysates of tumor and adjacent normal tissues from 3 patients with metastatic breast cancer and 2 patients with colorectal cancer. Differential expression of multiple checkpoint proteins, including BTLA, CD27, TIM-3, HVEM, CD40, GITR, LAG-3, CTLA-4, CD80/B7-1, PD-L1, or ICOS, were detected in these matched lysates, indicating the roles and complexity of checkpoint proteins in the tumor microenvironment. Our results demonstrate that the multiplex assays we developed are useful research tools for the simultaneous quantitation of immune checkpoint proteins, as well as its potential application in cancer biomarker discovery and translational research in cancer immunotherapy. **Please note, data is not shown in this poster for TLR-2.*

Methods

Sera, Cells, and Tissue: Human serum samples were purchased from Discovery Life Sciences, Inc. and BioreclamationIVT, including 20 colorectal cancer serum samples, 20 breast cancer serum samples, and 20 normal serum samples. All cell lines listed were obtained from ATCC. Human breast cancer and colorectal cancer tissue samples, and the corresponding adjacent normal breast or colorectal tissue samples were obtained from Asterand. Tissue lysates were prepared in MILLIPLEX® MAP Cell Signaling Lysis Buffer (Cat. No. 43-040).

Multiplex Protein Biomarker Immunoassays: Immune checkpoint protein biomarker profiles were quantitatively determined using the MILLIPLEX® MAP Immuno-Oncology Checkpoint Protein Panel (Cat. No. HCKPMAG-11K). Samples were analyzed on a Luminex® 200™ System with MILLIPLEX® Analyst 5.1 software.



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Results

Figure 1. Human Immuno-Oncology Checkpoint Protein Panel

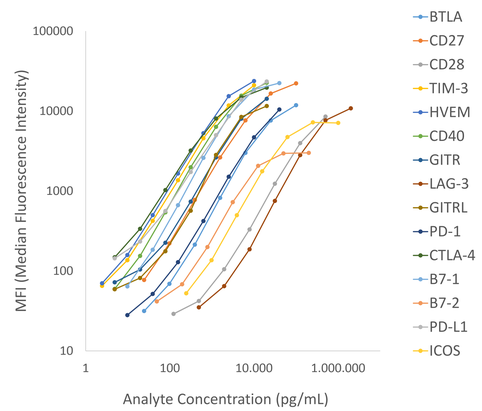


Figure Legend: **Figure 1. Human Immuno-Oncology Checkpoint Protein Panel Standard Curves.** The panel was designed based on the functions and interactions of the family members. *Please note, data is not shown in this poster for TLR-2.*

Figure 2. Soluble TIM-3 and sLAG-3 Levels in Sera from Breast Cancer and Colon Cancer Patients and Healthy Controls (n=20)

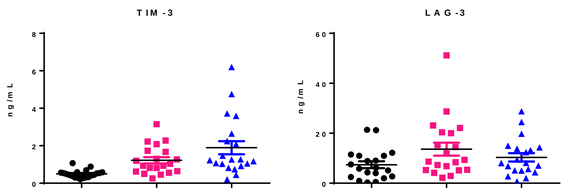


Figure 3. Soluble HVEM, sCD40 and sPD-L1 in Conditioned Media of 3 Breast Cancer Cell Lines (in A, B, C) and sHVEM in Conditioned Media of 3 Colon Cancer Cell Lines (in D)

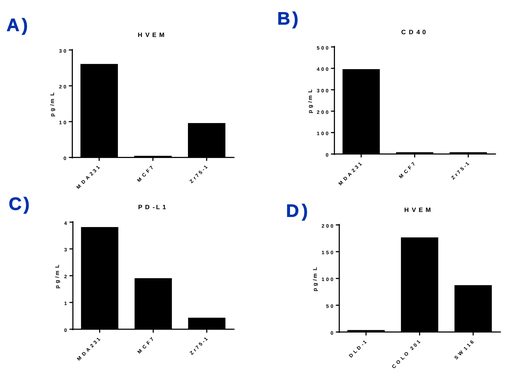
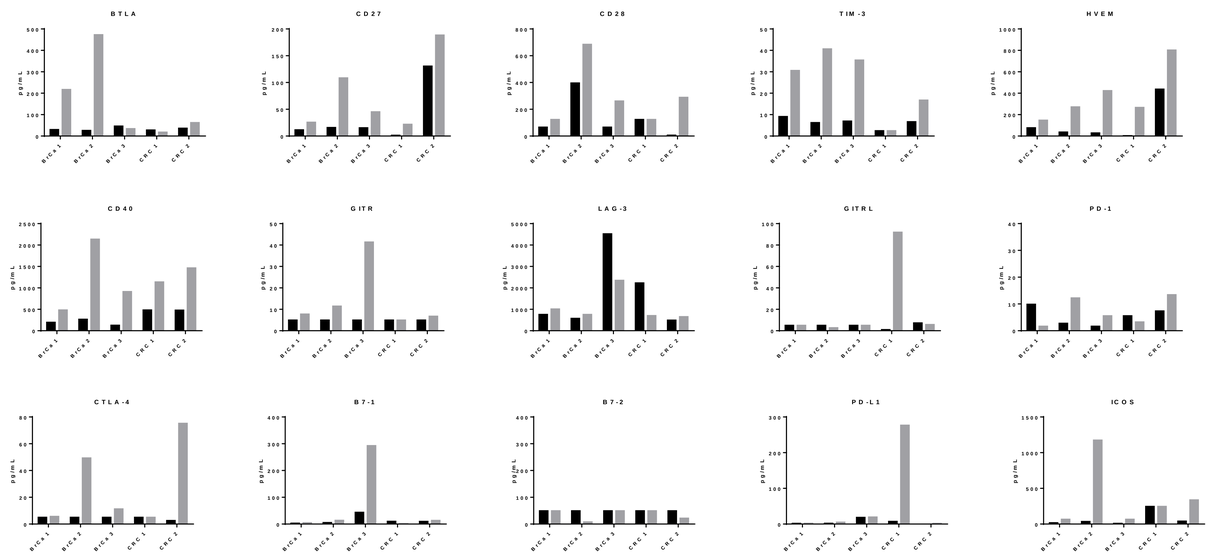


Figure 4. Immune checkpoint profiling in lysates of tumor and adjacent normal tissues from patients with breast cancer (n=3) and with colon cancer (n=2)

A)

		BTLA	CD27	CD28	TIM-3	HVEM	CD40	GITR	LAG-3	GITRL	PD-1	CTLA-4	B7-1	B7-2	PD-L1	ICOS
BrCa Pt 1	Normal	4.9	3.5	6.0	3.2	6.2	7.6	2.3	9.6	2.3	3.3	2.3	1.4	5.6	0.5	4.0
	Tumor	7.8	4.7	6.9	4.9	7.2	8.9	2.9	10.0	2.3	0.6	2.5	2.0	5.6	0.6	6.0
BrCa Pt 2	Normal	4.7	4.0	8.6	2.6	5.1	8.0	2.3	9.1	2.3	1.4	2.3	2.3	5.6	0.7	5.1
	Tumor	8.9	6.8	9.4	5.3	8.1	11.1	3.5	9.6	1.4	3.6	5.6	3.7	2.8	2.2	10.2
BrCa Pt 3	Normal	5.5	3.9	6.0	2.8	4.8	7.0	2.3	12.1	2.3	0.6	2.3	5.4	5.6	4.2	2.7
	Tumor	5.1	5.5	8.0	5.1	8.7	9.8	5.4	11.2	2.3	2.5	3.5	8.2	5.6	4.3	6.0
CRC Pt 1	Normal	4.8	0.1	6.9	1.3	0.3	8.9	2.3	11.1	-0.1	2.5	2.3	3.3	5.6	2.8	7.9
	Tumor	4.1	4.4	6.9	1.3	8.1	10.1	2.3	9.4	6.5	1.7	2.3	-0.7	5.6	8.1	7.9
CRC Pt 2	Normal	5.2	7.0	2.3	2.7	8.8	8.9	2.3	8.9	2.8	2.9	1.3	3.2	5.6	-2.7	5.3
	Tumor	5.9	7.6	8.2	4.1	9.6	10.5	2.8	9.3	2.5	3.7	6.2	3.7	4.4	0.5	8.4

B)



A) A heat map analysis of 15 checkpoint proteins in extracts of human tumor tissues and the matched adjacent normal tissues. The data is presented as log2 of the concentration in a Red-Yellow-Blue heat map showing higher values in Red and lower values in Blue (ND: not detectable; BrCa: breast cancer; CRC: colorectal cancer).

B) Differential expression of individual checkpoint proteins in breast or colon cancer tumor tissue relative to its adjacent normal tissue. Lysate samples (2 mg/mL) were prepared in MILLIPLEX® MAP Lysis Buffer and diluted in MILLIPLEX® MAP Assay Buffer before analysis. The Human Immuno-Oncology Checkpoint Protein Panel was used to quantitatively analyze cellular checkpoint proteins. Black bar: tumor tissue; Grey bar: adjacent normal tissue.

Summary

- We developed a human multiplex immunoassay panel for simultaneously quantifying 15 immune checkpoint proteins using 25-50µL of appropriately diluted samples for immuno-oncology research (Cat. No. HCKPMAG-11K, which also includes an anti-tumor immune regulator, TLR-2). *Please note, data is not shown in this poster for TLR-2.*
- MILLIPLEX® MAP profiling of these immune checkpoint proteins has identified secreted and cellular checkpoint protein biomarker candidates in cancer patient sera, tumor cell conditioned media, and human tumor tissue.
- We identified 2 putative circulating cancer biomarkers, sTIM-3 and sLAG-3.
  1) sTIM-3 is elevated in human colon cancer patient sera and breast cancer sera.
  2) sLAG-3 is elevated in human breast cancer sera when compared to the expression levels in sera from healthy controls.
- Differential expression of multiple checkpoint proteins were detected in breast and colon cancers.
  1) As soluble proteins harvested from tumor cell condition media.
  2) As cellular proteins in the lysates of tumor and adjacent normal tissues collected from patients.
- Our results showed the MILLIPLEX® MAP Human Immuno-Oncology Checkpoint Protein Panel (Cat. No. HCKPMAG-11K) is an ideal tool for studying immuno-oncology in cancer research. It allows simple, sensitive, reliable, and accurate biomarker identification/characterization and quantitative profiling of biologically important changes.

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