Multiplex immunoassay characterization of 48 cytokines, chemokines, and growth factors in colorectal cancer

Wen-Rong Lie, Harold Steiner, Sasha Williams, Munmun Banerjee, Anthony Saporita, Brooke Gilliam, Xiao Qiang

Introduction

Cytokines, chemokines, and growth factors are critical mediators of immune system function capable of signaling through autocrine, paracrine, and endocrine mechanisms. Their pleiotropic immunomodulatory properties allow these biomolecules to react to diverse stimuli and regulate the immune response. Many of these proteins have been proposed as cancer biomarkers and as key regulators of the tumor microenvironment (TME). Following the development of a Luminex® technology-based MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A 48-plex (Cat. No. HCYTA-60K) for profiling of 48 key immune mediators, we have developed another novel magnetic bead-based multiplex immunoassay panel, MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel B 48-plex (Cat. No. HCYTB-60K), for the simultaneous quantitation of additional 48 key immune proteins.

Colorectal cancer (CRC) is frequently characterized by chronic inflammation driving disease progression, with the TME serving as a potent reservoir from which the cytokines, chemokines, and growth factors exert their influence. In this study, we have performed protein biomarker analysis with the 48-plex Panel B immunoassay in serum CRC samples and the normal healthy controls. Of the 48 Panel B analytes tested in serum samples, BAFF, sFas, SCF, and MPIF-1 were observed to be elevated in CRC samples compared to healthy controls; and IL-20, ENA-78, and 6CKine were decreased in CRC samples compared to health controls. In addition, here we report the use of this novel 48-plex panel in other types of samples, including CSF, urine, milk, saliva, tumor lysates, and exosomal isolates, for establishing a profile for these proteins in each sample type. Lastly, we have applied both Panel A and Panel B to profile 96 human cytokines, chemokines, and growth factors in the study of tumor tissue lysate CRC samples and tissue lysates from the matched colorectal normal tissue.

Altogether, this study uses a novel multiplex immunoassay to characterize the immunological profile of colorectal cancer serum, focusing on 48 cytokines, chemokines, and growth factors. Additionally, alternative sample types were evaluated to establish the utility of this research tool for applications beyond serum, plasma, and cell culture supernatant.

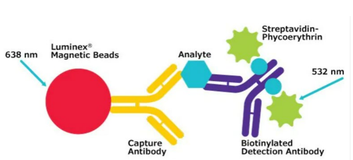
Methods

Samples Human colorectal cancer serum samples and normal healthy controls were purchased from Discovery Life Sciences, Inc. and BioIVT, including 9 colorectal cancer (CRC, n=9) serum samples, and 10 normal serum samples (Cont, n=10) for the serum sample testing. Human serum samples for exosome isolations were purchased from Discovery Life Sciences, Inc. and BioIVT, including 4 pancreatic cancer samples (n=4), and 4 normal controls (n=4) for the exosome sample testing. Circulating exosomes were isolated from serum samples using the Total Exosome Isolation kit (ThermoFisher). Human CRC tumor tissue samples, and the corresponding adjacent normal colorectal tissue samples were obtained from Asterand. All tissue lysates and exosomal lysates were prepared in MILLIPLEX® Cell Signaling Lysis Buffer (Cat. No. 43-040). The total protein concentrations in tumor lysates were determined using the Micro BCA™ assay. Human healthy control urine (n=10), saliva (n=10), milk (n=10), and 8 diseased cerebrospinal fluid [AD CSF (Alzheimer's Disease) n=4, MCI CSF (Mild Cognitive Impairment) n=4] samples were purchased from BioIVT.

Multiplex Immunoassays and Data Analysis Human cytokine, chemokine, growth factor biomarker profiles in normal and colorectal cancer serum, urine, saliva, milk, and CSF samples were quantitatively determined using MILLIPLEX® Human Cytokine/Chemokine /Growth Factor Panel B (Cat. No. HCYTB-60K). Human cytokine, chemokine, growth factor biomarker profiles in colorectal cancer tissue lysates and adjacent normal colorectal tissue lysates were quantitatively determined using both MILLIPLEX® Human Cytokine /Chemokine /Growth Factor Panel A (Cat. No. HCYTA-60K) and the novel MILLIPLEX® Human Cytokine /Chemokine /Growth Factor Panel B (Cat. No. HCYTB-60K). Samples were analyzed on a Luminex® 200™ System with MILLIPLEX® Analyst 5.1 software. Data analysis was performed for all immunoassays using the Belysa® Immunoassay Curve Fitting Software (Cat. No. 40-122). Figures were prepared in GraphPad Prism.

MILLIPLEX® Immunoassay Format MILLIPLEX® immunoassays use magnetic microspheres (beads) conjugated to capture antibodies. Each set of beads is distinguished by different ratios of two internal dyes yielding a unique fluorescent signature to each bead set, allowing researchers to simultaneously measure the analytes targeted by the capture antibodies. Native protein is analyzed by means of a "sandwich" immunoassay, pairing the capture beads with a biotinylated detection antibody.

Luminex® 200™ System

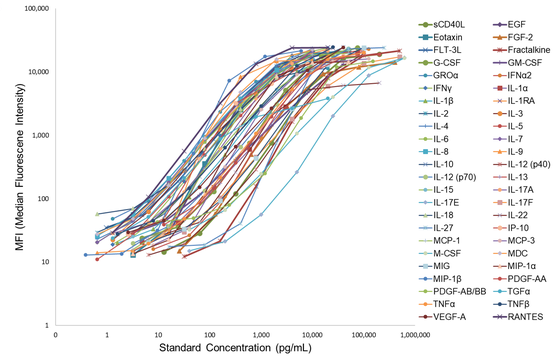


xMAP® INTELLIFLEX® Instrument

Results

Figure 1. The standard curves for Human Cytokine/Chemokine/Growth Factor Panel A and Panel B: Bead-based 48-plex immunoassays

(i) Human Cytokine/Chemokine/Growth Factor Panel A (Cat. No. HCYTA-60K) Standard Curves



(ii) Human Cytokine/Chemokine/Growth Factor Panel B (Cat. No. HCYTB-60K) Standard Curves

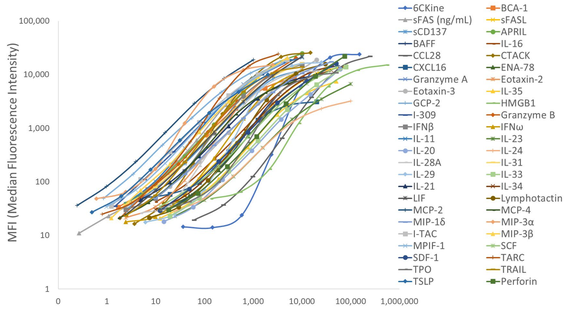
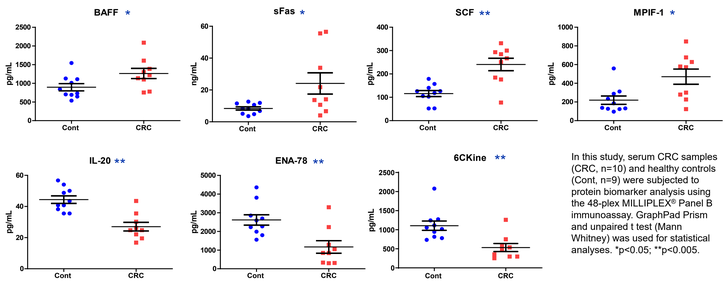


Figure 2. Differential expression of circulating serum BAFF, sFas, SCF, MPIF-1, IL-20, ENA-78, and 6Ckine in human colorectal cancer samples



In this study, serum CRC samples (CRC, n=10) and healthy controls (Cont, n=9) were subjected to protein biomarker analysis using the 48-plex MILLIPLEX® Panel B immunoassay. GraphPad Prism and unpaired t test (Mann Whitney) was used for statistical analyses. *p<0.05; **p<0.005.

Figure 3. Human Cytokine/Chemokine/Growth Factor Panel B utility for quantitative profiling 48 biomarkers in urine, CSF, saliva, and milk

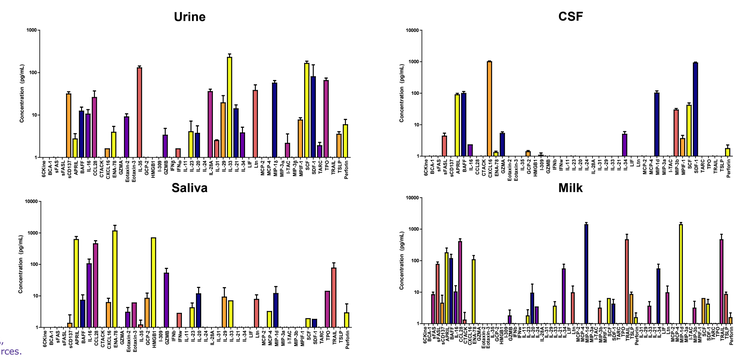
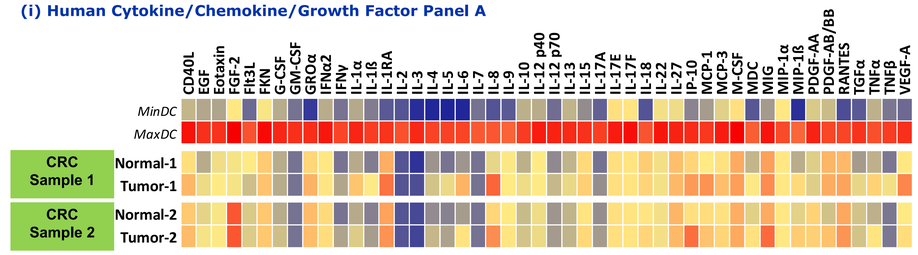


Figure 4. Simultaneously profiling 96 cellular proteins in the tumor tissue lysates in human colorectal cancer samples

A heatmap analysis of 96 cytokines, chemokines, growth factors in tissue lysates from tumor tissues and adjacent normal tissues from CRC samples (n=2). The data is presented as log2 of the concentration in a Red-Yellow-Blue heatmap showing higher values in Red and lower values in Blue. (i) Panel A 48-plex and (ii) Panel B 48-plex.

(i) Human Cytokine/Chemokine/Growth Factor Panel A



(ii) Human Cytokine/Chemokine/Growth Factor Panel B

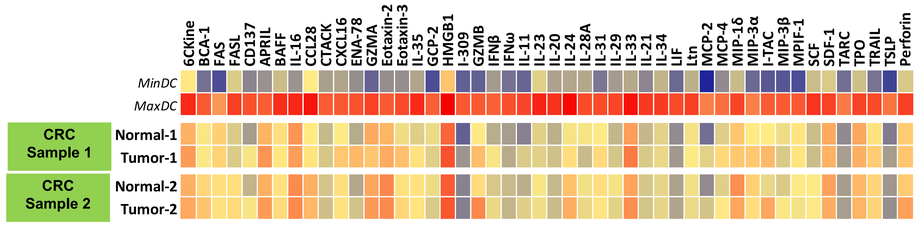
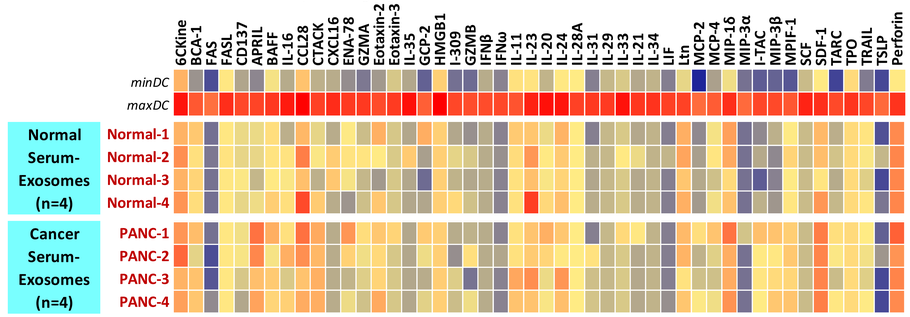


Figure 5. Profiling 48 exosomal cytokine, chemokines, and growth factors (Panel B) in the serum-derived exosomes in human pancreatic cancer samples

A heatmap analysis of Panel B 48 cytokines, chemokines, growth factors in serum-derived exosomal lysates isolated from pancreatic cancer sera (PANC, n=4) and normal healthy controls (Normal, n=4). The data is presented as log2 of the concentration in a Red-Yellow-Blue heatmap showing higher values in Red and lower values in Blue.



Summary

- In addition to the established MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A 48-plex, we have developed a second 48-plex, MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel B, for simultaneously quantifying unique immune proteins using 25 µL of diluted samples.
- MILLIPLEX® multiplex profiling of these 48 cytokines, chemokines, and growth factors in Human Cytokine/Chemokine/Growth Factor Panel B has identified secreted and cellular cancer-immunity biomarker candidates in colorectal cancer (CRC) sera, tumor cell conditioned media (data not shown), and human tumor tissue lysates.
- We reported 7 putative circulating cancer biomarkers in human CRC sera: BAFF, sFas, SCF, and MPIF-1 are elevated in human colorectal cancer sera when compared to the expression levels in sera from healthy controls, and IL-20, ENA-78, and 6CKine are reduced in human CRC sera when compared to the expression levels in sera from healthy controls.
- Differential expression of multiple cytokines, chemokines, and growth factors are detected as cellular proteins in tumor lysates and the matching adjacent normal tissue lysates from the same sample groups.
- Differential expression of exosomal cargo proteins were also demonstrated in serum-derived circulating exosomes isolated from cancer samples, as compared with the exosomes isolated from healthy controls.
- Our results show the addition of this new 48-plex MILLIPLEX®, Cytokine/Chemokine/Growth Factor Panel B (Cat. No. HCYTB-60K) to the 48-plex Human Cytokine/Chemokine/Growth Factor Panel A (Cat. No. HCYTA-60K) allows simple, sensitive, reliable, and accurate biomarker identification/ characterization and quantitative profiling of biologically important changes.

Learn more at SigmaAldrich.com/milliplex-cytokines