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tech note 6330

Profiling of Cell Signaling Pathways and Angiogenesis Biomarkers in Human Cancer Tissue

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Introduction

Cancer is a diverse family of diseases characterized by abnormal cells that divide uncontrollably and have the ability to infiltrate and destroy normal body tissues and organs (Mayo Clinic website, 2013). Cancer cells accumulate in tissues to form solid tumors, which stimulate blood vessel growth in a process called angiogenesis. These new vessels are believed to facilitate tumor expansion, as well as metastasis — which is the spread of cancer from its original location to other organs in the body.

Since the early 1970's, there has been a tremendous effort by the life science research community to eradicate cancer as a major life-threatening disease, yet cancer remains the second leading cause of death in the United States (CDC 2010). Thus, researchers in academic, pharmaceutical, and drug discovery settings have ample motivation to continue studying the pathogenesis of cancer with the goal of developing safer, more effective, and improved target treatments.

To help cancer researchers investigate key pathways involved in angiogenesis, metastasis, cell proliferation, apoptosis, inflammation, and other cancer-related processes, we developed the Bio-Plex™ Pro cell signaling assays and the Bio-Plex Pro human cancer biomarker panels (Peretz et al. 2012, Zimmerman et al. 2011, Zimmerman et al. 2012). The cell signaling assays enable highly sensitive detection of phosphoproteins and total target proteins in cell and tissue lysates, while the cancer biomarker panels allow robust quantification of soluble proteins in serum, plasma, cell culture media, and lysates. Bio-Plex Pro assays are based on Luminex xMAP technology (Dale et al. 2008, bulletin 5404) employing a magnetic bead–based workflow to enable multiplex detection of proteins in a single well of a 96-well microplate.

Here we describe a method to prepare homogenates from human tissues, as well as the successful application of these Bio-Plex Pro assays in measuring potential therapeutic targets and disease-related biomarkers in breast cancer samples.

Materials

Tissues were purchased from Asterand, a supplier of human tissues and biofluids, with accompanying clinical information. Three breast tissues were tested: normal (autopsy), HER-2-negative (HER-2(–)) tumor, and HER-2-positive (HER-2(+)) tumor tissues (surgical). Breast tumor tissues were diagnosed as infiltrating ductal carcinoma, and HER-2 protein expression was determined using Dako's HercepTest, an FDA-approved semiquantitative immunohistochemistry assay.

Bio-Plex Pro cell signaling assays and Bio-Plex Pro human cancer biomarker panels 1 and 2 were used to determine the levels of biomarkers in these tumors. The cell signaling assays include 23 assays for the detection of phosphorylated targets and 11 assays for total targets (i.e., both phosphorylated and unphosphorylated proteins). Human cancer biomarker panels 1 and 2 consist of 16 and 18 markers, respectively, with direct relevance to the pathogenesis of tumor-associated angiogenesis and related processes.

Methods

Tissue lysates were prepared as described previously (Gao et al. 2005); see Figure 1. Briefly, 100–200 mg of tissue was rinsed twice with ice-cold Bio-Plex cell signaling wash buffer and ground with a Dounce Tissue 2 ml Grinder containing 0.5–1.0 ml cell lysis buffer supplemented with Bio-Plex cell lysis factor QG and PMSF to a final concentration of 2 mM. After one freeze-thaw cycle, the lysate was pulse-sonicated 18 times with a Branson Sonifier 450 with a duty cycle of 40 and an output of 1. Cell debris was cleared by centrifugation at 16,000 × *g* for 10 min at 4°C. The supernatant was transferred to a clean tube, protein concentration was measured using the Bio-Rad DC™ protein assay, and aliquots were stored at –70°C until quantification using the Bio-Plex assays.

For the Bio-Plex Pro cell signaling assays, the lysate was diluted to 200 µg/ml with lysis buffer, and for the cancer biomarker panels, lysates were diluted with sample diluent HB plus 0.5% BSA. Because testing for cytokines and cancer biomarkers necessitates dilution with sample diluent HB, we recommend using lysis buffer to standardize protein concentrations prior to dilution. This protocol ensures that all lysates share the same buffer composition at testing. A minimum of twofold lysate dilution to a final protein concentration of 50 to 500 µg/ml is recommended in the product manual for Bio-Plex Pro human cancer panels 1 and 2. A twofold dilution is the minimum required to reduce detergent concentration to an acceptable level. A higher dilution factor with sample diluent is preferred for lysate stock concentrations greater than ~2 mg/ml.

The principle of this 96-well plate format, magnetic bead–based assay is similar to a capture sandwich immunoassay (Zimmerman et al. 2012). The capture antibody-coupled beads are first incubated with tissue lysate followed by incubation with biotinylated detection antibodies. After washing away the unbound biotinylated antibodies, the beads are incubated with a reporter streptavidin-phycoerythrin conjugate (SA-PE). Finally, the beads are passed through the Bio-Plex instrument, which measures the fluorescence of the bound SA-PE on each bead. Measurements are expressed as median fluorescence intensity (MFI) for a given bead/target population. All assay incubations were performed at room temperature according to the assay instruction manual. All washes were performed using a Bio-Plex Pro wash station. Data acquisition was performed using Bio-Plex Manager™ software.

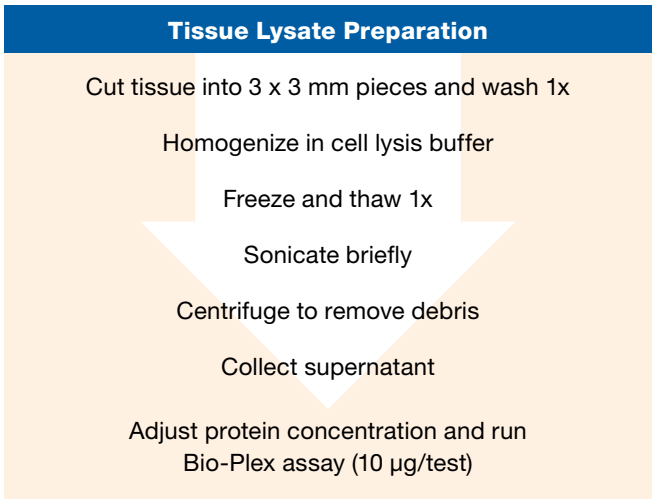

Fig. 1. Protocol for tissue lysate preparation.

Results

Breast cancer signaling pathways were evaluated with three panels of Bio-Plex Pro cell signaling assays. Three panels were custom mixed from the 23 available phosphoproteins, two 8-plex panels and one 7-plex panel as listed in Table 1; in total, 19 different proteins were assayed, some with multiple phosphorylation sites. Analyte groupings were selected to minimize cross reactivity. The assays were run twice, each in duplicate, with intra-assay and inter-assay coefficients of variation (%CV) of less than 20%.

Table 1. Cell signaling phosphoprotein assay panel analytes.

Panel A	Panel B	Panel C
Akt (Ser^{473})	Akt(Thr^{308})	HER-2 (Tyr^{1248})
c-Jun (Ser^{63})	EGFR (Tyr^{1173})	IGF-IR (Tyr^{1131})
EGFR (Tyr^{1068})	GSK-3α/β (Ser^{21}/Ser^{9})	IκB–α (Ser^{32}/Ser^{36})
Erk1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187})	JNK (Thr^{183}/Tyr^{185})	p38 MAPK (Thr^{180}/Tyr^{182})
MEK1 (Ser^{217}/Ser^{221})	NF-κB p65 (Ser^{536})	p70 S6 kinase (Thr^{389})
PDGFR–α (Tyr^{754})	P70 S6 Kinase (Thr^{421}/Ser^{424})	Smad2 (Ser^{465}/Ser^{467})
Stat1 (Tyr^{701})	PDGFR–β (Tyr^{751})	Stat3 (Ser^{727})
VEGFR-2 (Tyr^{1175})	Stat3 (Tyr^{705})	

The results presented in Figure 2 show that 12 of the 19 proteins tested underwent changes in phosphorylation compared with those in normal tissue, suggesting broad activation of signaling pathways. Consistent with the HercepTest immunochemical quantification of total HER-2 levels, we observed an increase in phosphorylated HER-2 in the HER-2(+) tissue, while levels in the tumor HER-2(–) tissue were unchanged in comparison with normal tissue. The human cancer biomarker panel 1, which detects total soluble HER-2, also detected higher levels in this tissue (Figure 3). We found that p-MEK1, p-Erk1/2, p-GSK-3α/β, p-Smad2, p-p70 S6 kinase, and p-Stat3 displayed higher levels in both cancer tissues, whereas the other markers showed tumor-specific phosphorylation patterns. For example, p-IκB-α was elevated in HER-2(–) tumor tissue, but the level remained unchanged in HER-2(+) tissue. Interestingly, of all the proteins tested, only p38 MAPK phosphorylation levels were lower in the HER-2(–) tissue. The significance of these changes in breast cancer requires further investigation and is outside the scope of this study. However, our data clearly demonstrate the applicability of Bio-Plex assays in the study of signaling pathways in cancerous tissue.

The data presented in Table 2 demonstrate the detection or nondetection of the 34 analytes comprising human cancer biomarker panels 1 and 2 in lysates of the four studied cell lines at total lysate protein concentrations of 20 or 100 µg/ml.

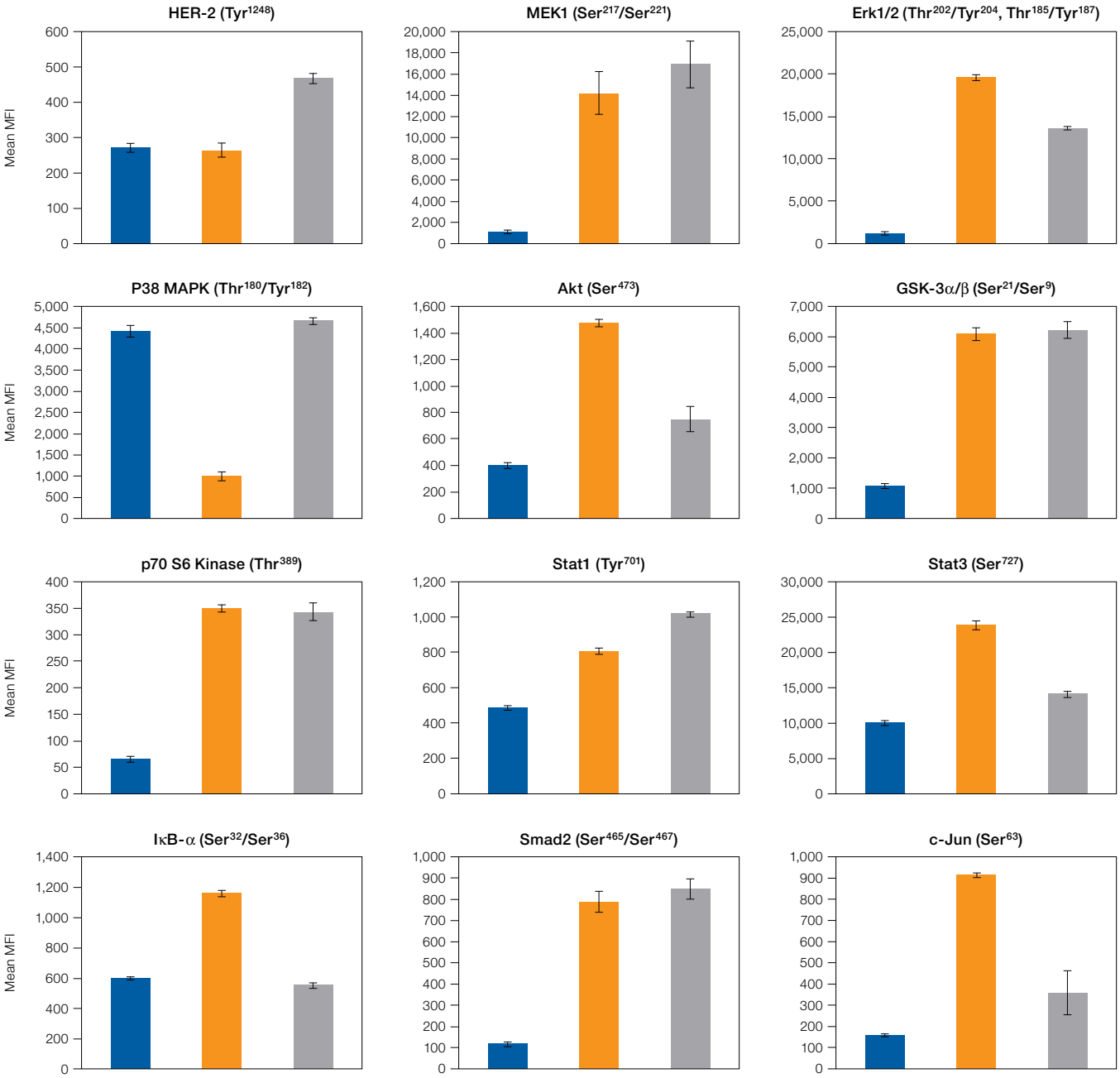

Fig. 2. Alternations in protein kinase profiling in breast cancer. Briefly, three Bio-Plex panels were used to simultaneously measure the levels of 19 phosphoproteins in 50 µl of sample per well at a lysate concentration of 200 µg/ml (i.e., 10 µg protein/well). MFI data are presented as duplicate means run over two days with STDEV. Normal breast tissue (■); HER-2(–) tumor (■); HER-2(+) tumor (■).

Table 2. Protein levels (pg/ml) in lysates from various cell lines using cancer biomarker panels 1 and 2.

	Cell line			
	VEGF/HUVECs	IFN-α/Jurkat	UV/HEK293	UV/COS7
Panel 1				
sEGFR1*	5.6	5.0	58.4	204.6
FGF basic*	4.2	3.3	8.4	0.9
Follistatin*	20.8	23.4	24.5	4.6
G-CSF*	2.0	1.9	2.0	0.1
sHER-2*	117.3	3.5	220.1	34.9
HGF*	5.5	5.6	4.1	ND
sIL-6Rα*	1.0	0.9	0.6	0.3
Osteopontin*	6.3	73.4	63.2	ND
Leptin*	3.2	4.9	1.1	ND
PDGF-BB*	32.4	33.2	36.1	9.3
PECAM-1*	10,517.6	380.8	ND	ND
Prolactin*	36.0	28.8	82.8	ND
SCF*	0.3	0.3	0.1	ND
sTie-2*	271.3	80.9	88.9	ND
sVEGFR-1*	140.0	63.3	11.2	ND
sVEGFR-2*	341.0	13.9	4.7	1.7
Panel 2				
Angiopoietin-2**	194.6	ND	3.1	0.5
sCD40L**	22.6	14.6	2.9	3.0
EGF**	1.3	0.6	ND	0.2
Endoglin**	4,614.8	6.3	3.6	181.0
sFASL**	26.3	3.5	5.4	4.4
HB-EGF**	12.4	1.2	0.7	10.2
IGFBP-1**	14.4	2.2	0.8	1.3
IL-18**	0.8	0.6	0.8	0.4
IL-6**	20.4	0.8	0.3	0.2
IL-8**	10.8	0.3	1.3	0.2
PAI-1**	3,012.9	ND	ND	579.8
PLGF**	43.7	ND	1.1	0.6
TGF-α**	4.7	1.2	0.0	0.9
TNF-α**	1.9	0.4	0.3	0.4
uPA**	114.7	1.8	2.8	62.9
VEGF-A**	7,003.1	ND	27.4	63.9
VEGF-C**	127.3	ND	40.1	67.0
VEGF-D**	25.0	ND	2.5	ND

* Lysate Conc 20 µg/ml
** Lysate Conc 100 µg/ml
ND, not detected

Figure 3 illustrates the differences in protein levels in tumor lysates measured using Bio-Plex Pro human cancer biomarker panels 1 and 2. HER-2(+) tumors showed increased levels of follistatin, HGF, osteopontin, prolactin, sTie-2, sVEGFR-1, sVEGFR-2, PECAM-1, and sHER-2 (panel 1) and EGF, sCD40L, sFASL, TNF-α, IL-18, PLGF, IL-8, TGF-α, VEGF-C, and uPA (panel 2). Almost all of these analytes were also elevated in HER-2(–) tumors except EGF and IL-18, for which the levels remained similar. The analytes that exhibited downregulation in HER-2(+) tumors were sEGFR (panel 1) and VEGF-A, Angiopoietin-2, endoglin, IL-6, and IGFBP-1 (panel 2). HER-2(–) tumors demonstrated downregulation of the same analytes except VEGF-A and Angiopoietin-2 and showed additional downregulation of leptin and EGF. These results suggest that each tumor has a distinct proteomic profile that can be identified by measuring the levels of these biomarkers.

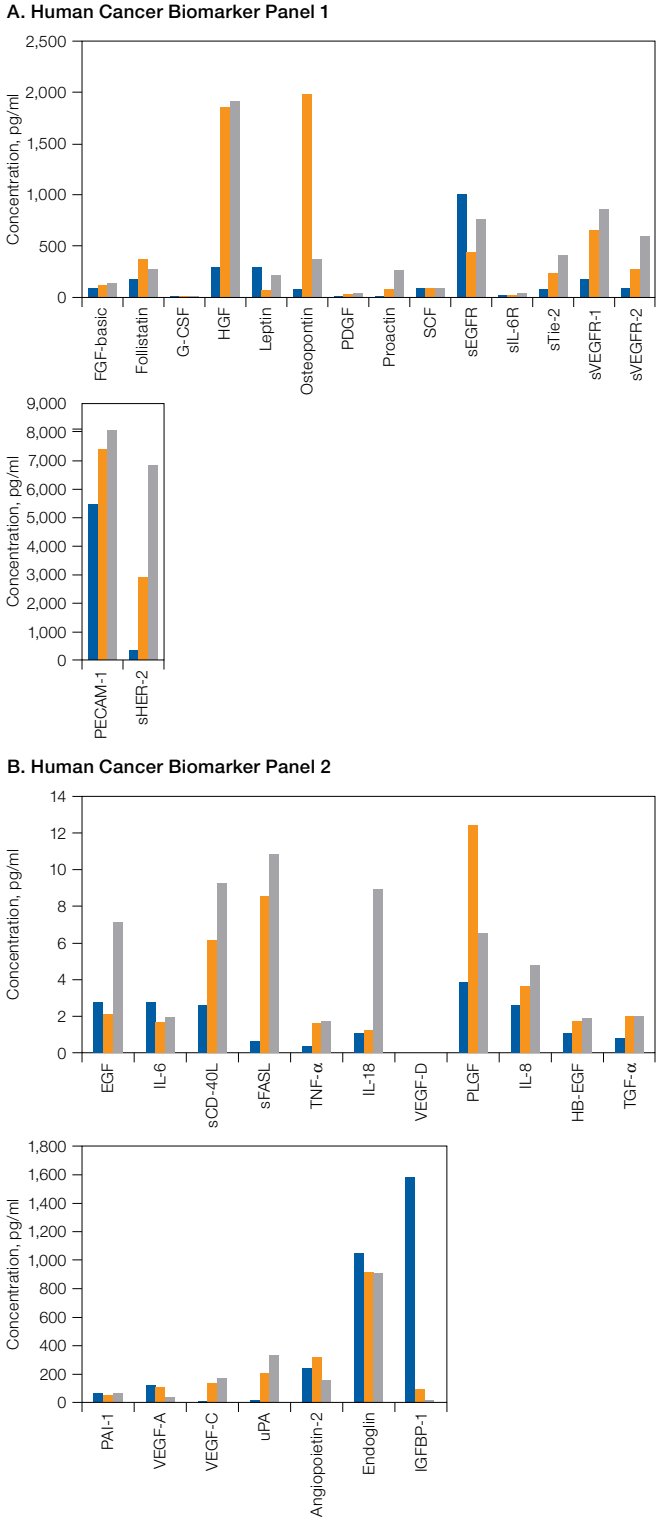

Fig. 3. Differences in the levels of markers measured using Bio-Plex Pro human cancer biomarker panels 1 (A) and 2 (B). Lysates diluted to 300 µg/ml in sample diluent HB with 0.5% BSA were added (50 µl) to each well. Results are reported as observed concentrations in pg/ml. Normal breast tissue (■); HER-2(–) tumor (■); HER-2(+) tumor (■).

Discussion

Despite the tremendous progress in identifying drug targets for cancer therapy, only 5–10% of the therapeutic candidates entering clinical trials progress beyond the early phases of clinical development (Doroshow and Parchment 2008). To improve productivity in this area, the FDA has suggested starting clinical efforts with smaller trials (Phase 0 studies) that measure biomarker responses demonstrating drug activity. The ability to detect multiple changes in cell signaling proteins, as well as biomarkers of angiogenesis, metastasis, and other processes specific to cancerous tissue uniquely positions the Bio-Plex platform as an important tool for investigating drug effects in clinical settings. Our assays were performed in multiplex, allowing a large number of measurements to be obtained in one test using only 4 µg of protein per well. Because needle biopsies extract only a small amount of tissue, such multiplexed assays present a great advantage over traditional methods such as ELISA.

Furthermore, with targeted therapies there is a need to select patients with tumors that will likely respond to drug therapy. Patient tumor types are typically classified by immunohistochemistry (IHC). Long considered the gold standard, this procedure is labor-intensive, time-consuming, and provides only a limited view of signal pathway circuits. The Bio-Plex platform, which yields many measurements from a single sample, can complement IHC to provide a broader view of cancer profiles and thus potentially improve patient selection. For example, using Bio-Rad Laboratories' Bio-Plex Pro assay panels, Alexopoulos et al. reported 6,500 measurements from one 96-well plate assayed for 17 phosphoproteins and 50 cytokines (Alexopoulos et al. 2010).

In summary, we demonstrated the application of three Bio-Plex panels for detecting proteins specifically involved in human cancer tissue. The newly configured assays were developed to provide a global view of cell signaling, angiogenesis, and other important cancer-related processes in one simple assay. These assays offer improved quantification, rapid interpretation, ease of use, high throughput, and reproducibility, making them widely applicable to both pre-clinical and clinical testing. In the clinic, the Bio-Plex Pro assays have already been used to assess responses to therapy and biomarker profiles in cancer patients (Dudek et al. 2009, Li et al. 2012), identifying correlations between biomarker levels and survival rates that demonstrated the effectiveness of a neoadjuvant regimen; thus, this multiplexed assay technology holds great potential for clinical oncology applications.

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Bulletin 6330 Rev A US/EG *12-1746 0213 Sig 1212*