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White Paper

Correlation of angiogenesis biomarkers with early metastatic progression in NSCLC, as determined using a multiplexed immunoassay kit

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About the Author

Dr. Jeffrey A. Borgia is an Associate Professor at Rush University Medical Center in Chicago, IL, in the Departments of Pathology and the Department of Cell and Molecular Medicine, and has been at Rush for 15 years. Dr. Borgia also holds the positions of Director of the Rush Biomarker Development Core as well as the Rush Translational Cancer Research Graduate Program. His research is focused on developing novel "-omics" diagnostic tests for profiling patient specimens and providing mechanistic insights for pathophysiology. Dr. Borgia oversees the collection and annotation of patient specimens for translational research projects and ongoing clinical trials at the Rush University Cancer Center. Additionally, at the Core, he and his team work on discovery and development of new/novel biomarkers for use in diagnostics.

Introduction

Angiogenesis is a fundamental process in growth, development, and tissue repair in which the underlying vascular infrastructure crucial to the delivery of nutrients is created by a well-studied combination of growth factors and regulatory elements. Dysregulation of this process leads to abnormal blood vessel growth involved in many common diseases, and plays a significant role in tumor growth and metastasis. In many types of cancer, aberrations in regulatory signaling pathways lead to sustained angiogenesis, a necessary component fueling the ability of cancerous tissue to thrive via continuous unregulated replication.[1,2] The process of angiogenesis is normally a tightly regulated balance between proangiogenic growth factors (VEGF, FGF, PDGF, TGF) and antiangiogenic signaling molecules (thrombospondin, angiostatin, and endostatin), along with complex membrane-bound receptors and endothelial cell interactions.[1,2] Malignant cells, however, sway this delicate equilibrium, resulting in rapid vascular growth with various underlying structural and functional abnormalities. From an investigative and therapeutic standpoint, it is important to identify and subsequently target the growth factors or regulatory elements that play crucial roles in allowing angiogenic dysregulation. With the overwhelming numbers of these targets for potential investigation, it is a challenge to decide among them and efficiently quantitate multiple targets.

Current detection assays are limited by minimal automation, low overall output and excessive cost. To quickly and affordably identify specific angiogenic processes, it is necessary to screen large panels of vascular analytes and growth factors with some level of automation or high throughput. Here, we demonstrate the utility of the MILLIPLEX® map Human Angiogenesis/Growth Factor Magnetic Bead Panel 1, based on the Luminex® xMAP® bead-based multiplexed assay platform, in the analysis of 17 targets involved in the angiogenesis pathway in a cohort of 38 patients with various stages of non-small cell lung cancer (NSCLC). Our objective was to determine if the MILLIPLEX® assay is able to accurately quantify known angiogenesis targets in a practical, high-throughput fashion.

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Methods

Patient Cohort

Serum was collected from a total of 38 patients with pathologically confirmed NSCLC between 2004 and 2008 at Rush University Medical Center in Chicago, IL. Full Institutional Review Board approval was obtained for this study, and patient consents are on record for all participants. Twelve patients were pathologically staged[3] as $T_{1-2}N_0M_0$ ("no metastatic progression"), 12 with $T_{1-3}N_{1-2}M_0$ ("locally-advanced"), and 14 patients with $T_{1-4}N_{1-2}M_1$ disease ("presence of distant metastases"). All patients were not previously treated with either chemo- or radiotherapy.

Sample Preparation

Serum was processed from whole blood using conventional methods within an hour of venipuncture and archived at -80 °C. No sample was subjected to more than two freeze/thaw cycles for this study. Frozen samples were thawed completely at 4 °C, mixed by vortexing, and centrifuged at 10,000xg for 10 minutes to remove particulates. All samples were diluted 1:3 in the provided assay buffer solutions, as recommended in the MILLIPLEX® map assay kit protocols.

Immunoassay Protocol

The Human Angiogenesis/Growth Factor Magnetic Bead Panel 1 (Cat. No. HAGP1MAG-12K) was used to quantify the following 17 human angiogenesis and growth factor biomarkers: Angiopoietin-2, BMP-9, EGF, Endoglin, Endothelin-1, FGF-1 (acidic FGF), FGF-2 (basic FGF), Follistatin, G-CSF, HB-EGF, HGF, IL-8, Leptin, PLGF, VEGF-A, VEGF-C, and VEGF-D. Antibody-conjugated magnetic beads were prepared by sonicating for 30 seconds, then vortexing 1 minute. All samples, quality control samples and standards were prepared as recommended in the MILLIPLEX® map assay kit protocols with supplied diluents, and processed in duplicate batches. 200 µL of assay buffer was added to each well and decanted. 25 µL of each sample and 25 µL of prepared beads were then added into appropriate wells, along with buffering solutions. The plate was subsequently sealed and incubated overnight at 4 °C. The plates were washed 3 times and followed by the addition of 25 µL detection antibodies into each well. After 1-hour incubation at room temperature, 25 µL of Streptavidin-Phycoerythrin was added to each well and incubated for an additional 30 minutes at room temperature.

The plates were washed 3 times and finally resuspended in 100 µL of sheath fluid in each well. The assay plate was then analyzed with the Luminex® instrument equipped with xPONENT® software. Statistical processing was accomplished in SPSS v15.0 and Microsoft Office Excel® 2007. Clinical outcomes were assessed by log-rank and Kaplan-Meier estimates of median time to recurrence using the R statistical software package.

Results and Discussion

We used the MILLIPLEX® map Human Angiogenesis/Growth Factor Magnetic Bead Panel 1 to quantify 17 analytes relevant to angiogenesis in clinical specimens representative of the different stages of disease progression in NSCLC. As seen in both **Table 1** and **Figure 1**, our results showed superb assay precision across a wide range of analyte concentrations and comparable performance characteristics in the patient samples, as shown in **Table 2**.

Based on the cohorts we tested, we observed significantly higher expression of IL-8, HGF and HB-EGF in serum from patients with "distant metastases"/disseminated disease, as compared to serum from patients with no metastases (**Table 2**, **Figure 2**). These trends were consistent with previous work produced by our laboratory, that varying expression of growth factors and other signaling molecules likely involved in the angiogenesis pathway exist and may predict tumor metastasis.[4]

Analyte	$\%CV_{mean}$
Angiopoietin-2	7.5
BMP-9	8.7
EGF	3.3
Endoglin	4.8
Endothelin-1	3.4
FGF-1	7.3
FGF-2	2.7
Follistatin	5.8
G-CSF	6.9
HB-EGF	7.2
HGF	11.2
IL-8	3.1
Leptin	4.6
PLGF	4.2
VEGF-A	7.5
VEGF-C	7.9
VEGF-D	2.9

Table 1. The %CVmean was calculated by averaging %CV values for each standard across the 8-point standard range (including 0 point). Levels for the angiogenesis biomarkers were determined based on the 7-point standard curve suggested by the MILLIPLEX® map assay kit protocol, and provided a wide dynamic range for each analyte (**Figure 1**). This dynamic range enabled most targets to be readily measured in human serum.

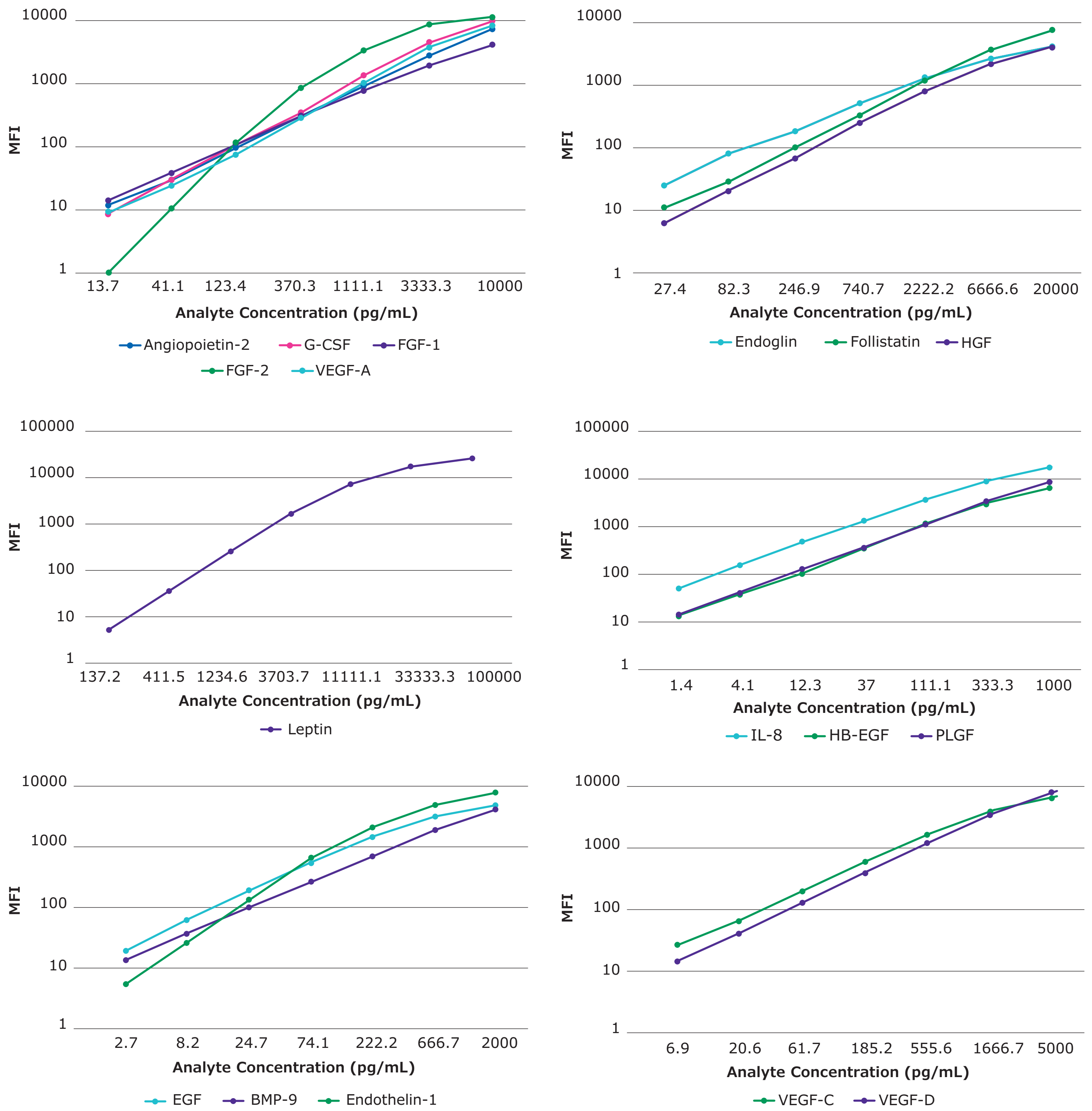

Figure 1. Standard curves for each analyte measured by the MILLIPLEX® map Human Angiogenesis/Growth Factor Magnetic Bead Panel 1. (Note: MFI = Median Fluorescence Intensity.)

	$T_{1-2}N_0M_0$		$T_{1-3}N_{1-2}M_0$		$T_{1-4}N_{1-2}M_1$	
	Range	Median	Range	Median	Range	Median
Angiopoietin-2	607 – 2,181	1,628.00	616 – 4,422	1398.00	770 – 14,250	1,816.00
BMP-9	56.7 – 352.6	98.30	15.9 – 480.2	110.40	15.9 – 428.3	162.90
EGF	3.34 – 62.18	17.6	3.34 – 153.39	38.77	3.3 – 76.4	12.53
Endoglin	203.6 – 2,112	613.7	96 – 1,074.6	649.20	236 – 2,398	497.93
Endothelin-1	2.6 – 12.7	8.19	2.6 – 113.2	2.59	2.6 – 10.9	5.24
FGF-1	0.21 – 19.0	4.71	0.21 – 8,404	2.41	0.21 – 54.0	4.00
FGF-2	41.1 – 330.3	41.10	41.1 – 425.7	41.10	41.1 – 133.2	41.10
Follistatin	175.0 – 2,052	848.40	204.9 – 2,824	839.70	228.2 – 1,353	599.10
G-CSF	2.80 – 108.8	4.26	2.80 – 91.4	2.80	2.80 – 370.8	2.80
HB-EGF	16.2 – 99.1	48.20	31 – 500.5	79.60	32.7 – 198.9	93.00
HGF	34.6 – 316.2	108.40	11.9 – 1,473.3	259.40	129.3 – 1960	253.40
IL-8	0.71 – 1.6	0.95	0.65 – 6.61	1.42	0.96 – 25.4	1.71
Leptin	10,620 – 51,383.4	14,265	1,572 – 24,840	6,585	1,241 – 36,763	9,554
PLGF	0.05 – 16.4	8.88	0.69 – 73.6	5.61	1.41 – 18.2	6.30
VEGF-A	4.5 – 770.5	43.80	15.4 – 1418.4	160.6	6.7 – 978.2	227.4
VEGF-C	1.63 – 65.5	27.40	1.63 – 1,264.1	47.20	1.63 – 61.5	35.30
VEGF-D	0.53 – 27.3	9.30	0.53 – 230.6	3.33	0.53 – 34.3	2.90

Table 2. Analysis of 3 cohorts of NSCLC patients with the MILLIPLEX® map Human Angiogenesis/Growth Factor Magnetic Bead Panel 1. (Note: All values are provided in pg/mL.)

When these measured values were evaluated by a Mann-Whitney Rank Sum test in relation to the clinical groups evaluated, we found that a number of biomarkers were correlated with progression of the disease to the locoregional lymph nodes (see **Figure 2**). Multiple markers (e.g., EGF, PLGF, VEGF-A, and VEGF-C) trended towards significance; however, significance was not reached due to the low number of patients involved in this study. Interestingly, the patterns observed only provided insights into early stages of metastatic progression and provided few insights into biological processes necessary for distant spread of the tumor.

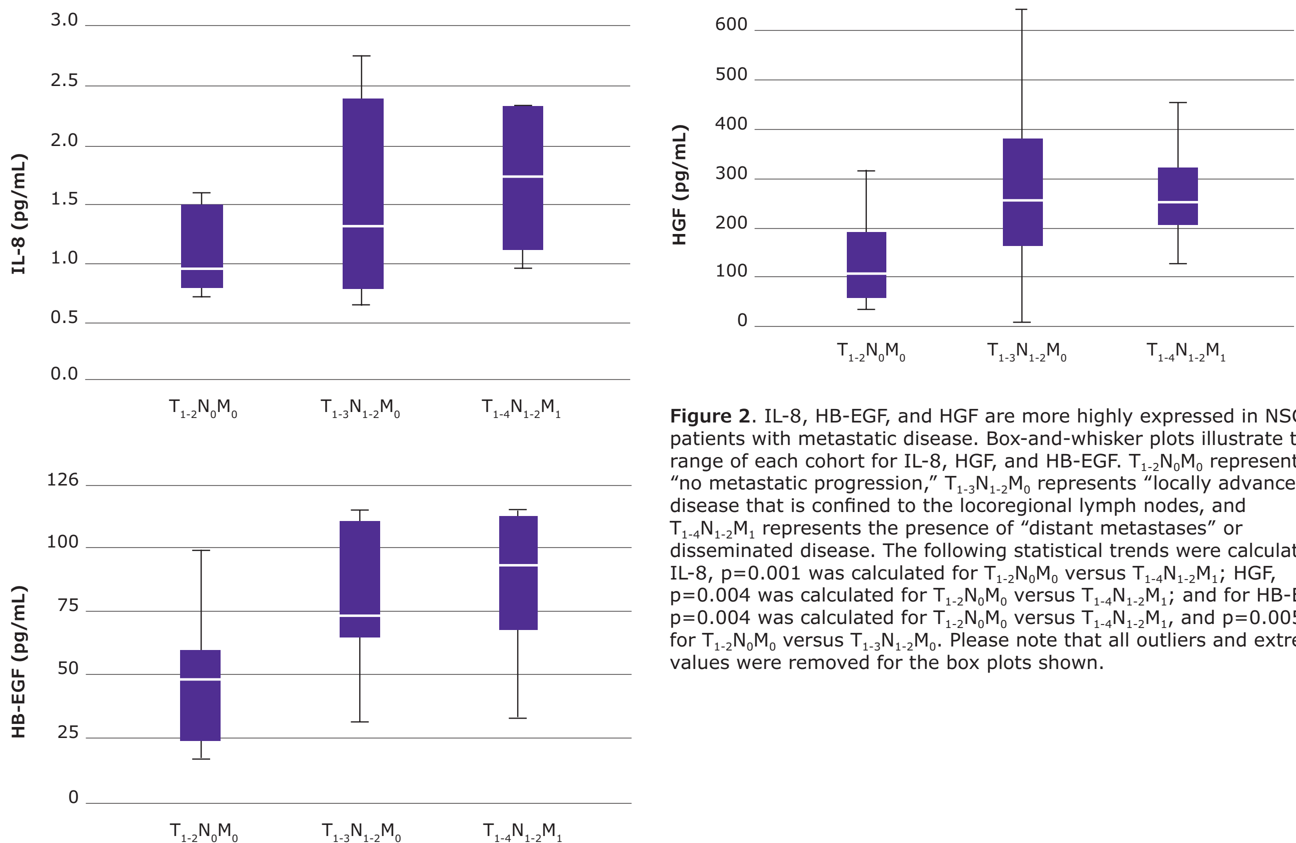

Figure 2. IL-8, HB-EGF, and HGF are more highly expressed in NSCLC patients with metastatic disease. Box-and-whisker plots illustrate the range of each cohort for IL-8, HGF, and HB-EGF. $T_{1-2}N_0M_0$ represents "no metastatic progression," $T_{1-3}N_{1-2}M_0$ represents "locally advanced" disease that is confined to the locoregional lymph nodes, and $T_{1-4}N_{1-2}M_1$ represents the presence of "distant metastases" or disseminated disease. The following statistical trends were calculated: IL-8, p=0.001 was calculated for $T_{1-2}N_0M_0$ versus $T_{1-4}N_{1-2}M_1$; HGF, p=0.004 was calculated for $T_{1-2}N_0M_0$ versus $T_{1-4}N_{1-2}M_1$; and for HB-EGF, p=0.004 was calculated for $T_{1-2}N_0M_0$ versus $T_{1-4}N_{1-2}M_1$, and p=0.005 for $T_{1-2}N_0M_0$ versus $T_{1-3}N_{1-2}M_0$. Please note that all outliers and extreme values were removed for the box plots shown.

Given the findings presented previously, we decided to focus on the $T_{1-2}N_0M_0$ and $T_{1-3}N_{1-2}M_0$ groups in terms of clinical outcome measures. Specifically, we evaluated the median time to recurrence in relation to biomarker concentrations using the median value for each biomarker as the threshold (see **Figure 3**). A total of 9 biomarkers were found with a log-rank p value less than 0.05. Not shown in **Figure 3** are BMP-9, Follistatin, HB-EGF, Angiopoietin-2, and VEGF-C. These data may provide important insights into clinically relevant biological changes that are mechanistically implicated in disease recurrence.

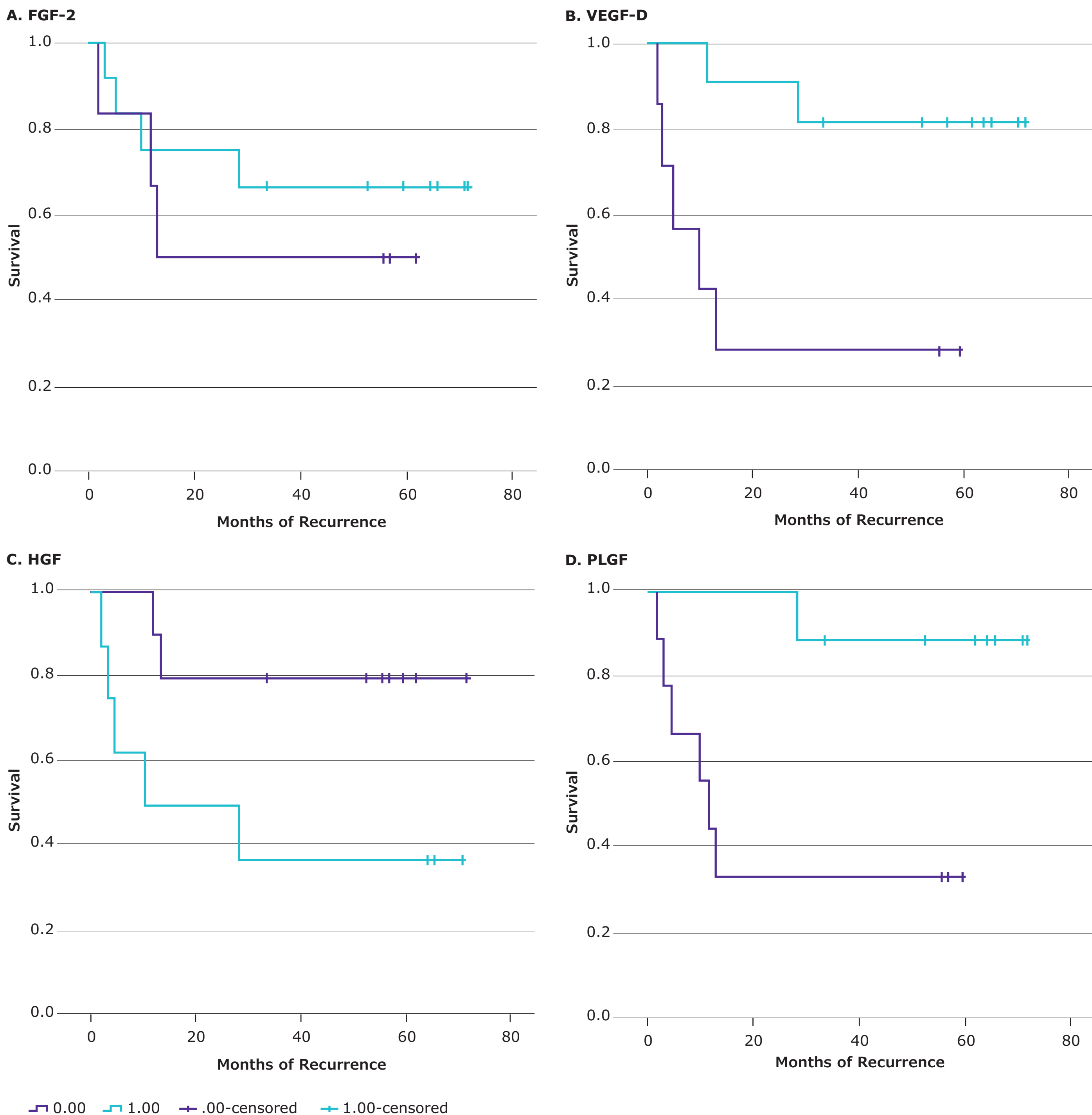

Figure 3. Kaplan-Meier estimates of the median time to recurrence. Clinical outcomes for the $T_{1-2}N_0M_0$ ("no metastatic progression") and $T_{1-3}N_{1-2}M_0$ ("locally advanced") groups were evaluated via log-rank analysis for each of the 17 biomarkers for the median time to recurrence. Shown above are the Kaplan-Meier curves for (A) FGF-2 [p=0.049], (B) VEGF-D [p=0.009], (C) HGF [p<0.001], and (D) PLGF [p=0.010]. Purple lines represent patients with values less than measured median concentrations, whereas the cyan lines represent values larger than the measured median concentrations.

Conclusion

These findings are illustrative of the general practical ability of this MILLIPLEX® map assay kit to generate information that can directly enhance studies of cancer metastasis. Because the angiogenesis pathway being studied here is so complex and with so many targets to analyze, simultaneous quantification of multiple analytes, such as that provided by the MILLIPLEX® map Human Angiogenesis/Growth Factor Magnetic Bead Panel 1, conserves valuable resources. This panel accurately measured multiple angiogenesis biomarkers relevant to our research. By selecting combinations of analytes within the panel, any researcher can easily customize the panel to answer specific research questions.

References

1. Winder, T., Lenz, H. Vascular endothelial growth factor and epidermal growth factor signaling pathways as therapeutic targets for colorectal cancer. Gastroenterology. 2010; 138(6): 2163 – 76.
2. Carmeliet, P., Jain, R.K. Molecular mechanisms and clinical applications of angiogenesis. Nature. 2011; 473(7347): 298 – 307.
3. Rami-Porta, R., Crowley, J.J., Goldstraw, P. The revised TNM staging system for lung cancer. Ann Thorac Cardiovasc Surg 200v9; 15(1): 4 – 9.
4. Farlow, E.C., Vercillo, M.S., Coon, J.S., et al. A multi-analyte serum test for the detection of non-small cell lung cancer. British Journal of Cancer 2010; 103(8): 1221.

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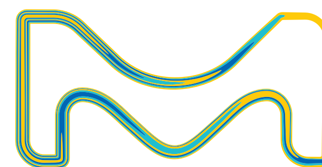

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