Detection of Hepatocellular Carcinoma Cases Using a Multiplex Cancer Biomarker Panel

Anu Mathew, Eli N. Glezer, Martin Stengelin, Mingyue Wang, and Jacob N. Wohlstadter

Meso Scale Diagnostics, LLC, Rockville, Maryland, USA

1 Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide with a 5-year survival rate of only 14% in the United States. The age-adjusted incidence rate of HCC in the United States is high and rapidly rising (from 1.6 per 100,000 in 1975 to 7.9 in 2009). The only clinically useful serum biomarker for HCC is alpha-fetoprotein (AFP), which has a reported sensitivity of 39% to 64% and specificity of 76% to 91%. This is neither sensitive nor specific enough to be useful, especially since most HCC cases are detected at an advanced stage when curative surgery is no longer possible.

In this study, the effectiveness of other cancer-related biomarkers for specific detection of HCC was evaluated using an electrochemiluminescence-based, multiplex serum/plasma immunoassay panel developed on the MSD® platform. An MSD MULTI-ARRAY® 10-plex assay panel (AFP, carcino-embryonic antigen [CEA], cancer antigen 125 [CA 125 or Muc-16], carbohydrate antigen 19-9 [CA 19-9, sialyl Lewis A], osteopontin [OPN], matrix metalloproteinase 9 [MMP-9], ErbB2, E-cadherin, soluble epidermal growth factor receptor [EGFR], and cKit) was used to screen 25 HCC, 25 cirrhosis (alcohol-induced or due to fatty liver disease), and 30 normal subject serum samples. The assay protocol was simple: a small volume of sample was diluted and added to blocked and washed plates. After a 2-hour incubation with agitation, plates were washed and detection antibody reagent was added. After a 1-hour incubation, plates were washed and read on an MSD SECTOR® Imager 6000 (read time 70 seconds).

The levels of AFP, CEA, CA 125, and CA 19-9 were found to be significantly elevated in the HCC samples compared to levels in the cirrhosis and/or normal samples. The levels of OPN, MMP-9, E-cadherin, and ErbB2 were also significantly altered in the different sample types. Some of these biomarkers, either alone or in combination, were better than AFP at distinguishing HCC patients from controls. Combinations of these biomarkers could provide superior performance compared to existing HCC detection modalities. The selected biomarkers must be further evaluated using different sample cohorts to determine effectiveness for detection of HCC cases, particularly in those with different developmental etiologies. Early detection of HCC in patients would enable therapeutic intervention at a stage where it would be most effective, significantly reducing mortality rates for HCC, one of the few cancers showing increasing incidence in the United States.

2 Methods

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY and MULTI-SPOT® microplates.

Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

Protocol

1. Dilute serum samples 5-fold in calibrator diluent.
2. Add 150 µL/well of Blocker A solution and incubate for 1 hour at room temperature (RT).
3. Wash with PBS-T. Add 25 µL/well of diluted sample/standard and 25 µL/well assay diluent. Incubate for 2 hours at RT.
4. Wash with PBS-T. Add 25 µL/well detection antibody. Incubate for 1 hour at RT.
5. Wash with PBS-T. Add 150 µL of Read Buffer T. Read plate on MSD imager.

3 HCC and Cirrhosis Sample Information

Cirrhosis Patient Samples

Etiology	Gender	Age
Alcohol Induced	Male	54
Alcohol Induced	Male	52
Alcohol Induced	Female	41
Alcohol Induced	Male	43
Alcohol Induced	Male	49
Alcohol Induced	Male	50
Alcohol Induced	Male	57
Alcohol Induced	Female	49
Alcohol Induced	Female	53
Alcohol Induced	Male	45
Alcohol Induced	Male	53
Alcohol Induced	Female	61
Alcohol Induced	Male	55
Fatty Liver Disease Induced	Female	83
Fatty Liver Disease Induced	Female	50
Fatty Liver Disease Induced	Male	56
Fatty Liver Disease Induced	Male	52
Fatty Liver Disease Induced	Male	60
Fatty Liver Disease Induced	Male	59
Fatty Liver Disease Induced	Male	56
Fatty Liver Disease Induced	Male	72
Fatty Liver Disease Induced	Male	48
Fatty Liver Disease Induced	Female	69
Fatty Liver Disease Induced	Male	65
Fatty Liver Disease Induced	Male	58
	Average Age	56
	Median Age	54

HCC Patient Samples

Medications	Stage	Gender	Age
None	2	Female	46
None	2	Female	52
None	2	Male	57
None	2	Male	58
None	2	Female	38
None	2	Female	77
None	2	Female	62
5FU	2	Female	49
5FU	3	Female	50
5FU	3	Female	47
5FU	3	Female	48
5FU	3	Female	65
Avastin, 5FU	2	Female	74
Avastin, 5FU	2	Male	63
Avastin, Carboplatin	2	Female	62
Carboplatin	2	Male	71
Carboplatin	2	Female	61
Gemzar	4	Female	66
Gemzar	2	Male	57
Gemzar	2	Female	58
Zometa	3	Female	47
Zometa	3	Female	78
Zometa	2	Male	63
Zometa	2	Male	67
Zometa, 5FU	3	Female	74
		Average Age	61
		Median Age	63

Table 1. HCC and cirrhosis sample details. Serum samples collected from patients diagnosed with cirrhosis or with HCC were obtained from a commercial source. These samples were tested with the 10-plex assay along with 30 samples from normal, age-matched donors.

4 Performance of Individual Biomarkers

The ability of the individual markers AFP, CEA, CA 125, CA 19-9, OPN, MMP-9, E-cadherin, and ErbB2 to detect primary HCC and/or cirrhosis is summarized in Table 2 as AUC (area under the curve) values from ROC (receiver operating characteristic) analysis. Higher values indicate better ability to distinguish between the listed sample types. The levels of AFP, CEA, CA 125, and CA 19-9 were found to be significantly elevated in the HCC samples as compared to levels in the cirrhosis and normal samples. The performance of the CA 125, CA 19-9, and CEA assays were comparable, if not superior, to performance of AFP in distinguishing HCC samples from normal samples, HCC samples from cirrhosis samples, and/or cirrhosis samples from normal samples. The levels of OPN, MMP-9, E-cadherin, and ErbB2 were also significantly altered in the different sample types and showed utility in distinguishing HCC and cirrhosis samples from normal samples.

	AUC Values from ROC Curves							
	E-cadherin	ErbB2	AFP	CA 125	CA 19-9	CEA	MMP-9	OPN
HCC/normal	0.72	0.85	0.92	0.88	0.95	0.93	0.93	0.89
HCC/cirrhosis	0.16	0.54	0.85	0.92	0.93	0.92	0.67	0.28
Cirrhosis/normal	0.86	0.78	0.76	0.87	0.81	0.54	0.82	0.91

Table 2. Analysis of each biomarker's ability to distinguish HCC samples from normal samples (HCC/normal), HCC samples from cirrhosis samples (HCC/cirrhosis), and cirrhosis samples from normal samples (Cirrhosis/normal).

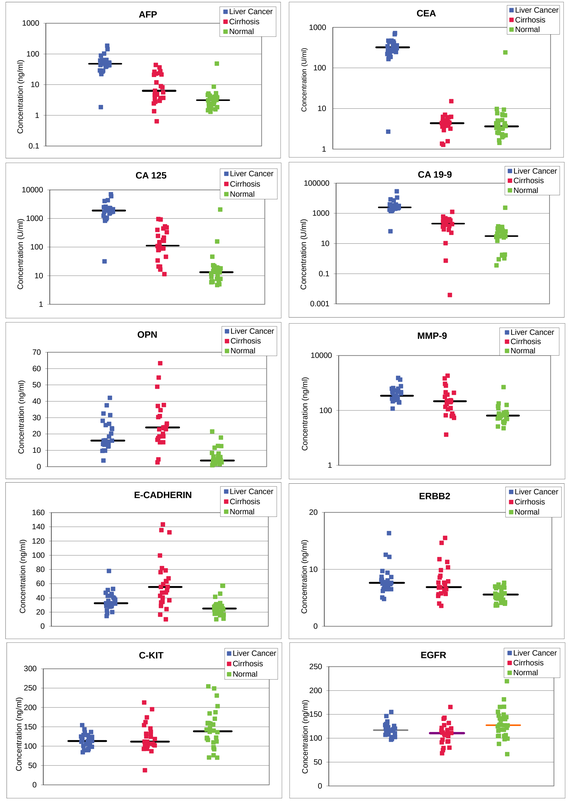
The data for the top 4 markers (AFP, CA 19-9, CA 125, CEA) were also used to derive scores of normalized mean differences between the 3 groups of samples as another means of determining the relative specificity of the markers in distinguishing the groups (Table 3). Scores with a magnitude ≥ 1 indicate that the biomarker has utility in distinguishing between 2 classes of samples, with increasing scores (e.g., scores with magnitudes ≥2) indicating increasing discriminating ability. According to this analysis, CA 19.9 performs almost as well as AFP in discriminating HCC from cirrhosis and from normal samples, while CA 125 and CEA perform better than AFP in both categories. CEA had scores >3 for distinguishing HCC from cirrhosis and from normal samples, indicating that CEA is a highly discriminatory marker for specific detection of HCC cases in patient populations that potentially include patients with cirrhosis.

	AFP	CA 19-9	CA 125	CEA
HCC/normal	3.6	3.0	4.6	4.5
HCC/cirrhosis	1.8	1.5	2.6	5.9
Cirrhosis/normal	0.7	0.8	1.8	0.3

Table 3. Normalized mean difference scores were calculated for each biomarker as $[(D - N)/((\sigma_D+\sigma_N)*0.5)]$ for HCC versus normal, HCC versus cirrhosis, or cirrhosis versus normal samples. D = mean concentration of biomarker for case sample set, N = mean concentration of biomarker for control sample set (LOG transformed data). σ_D and σ_N = standard deviation of concentrations for case or control sets (LOG transformed data), respectively.

5 Individual Sample Distribution for Each Marker

Biomarker concentrations in HCC (blue), cirrhosis (red) (n=25 each), and normal (green) (n=30) samples are shown, with median values indicated for each sample group (—).

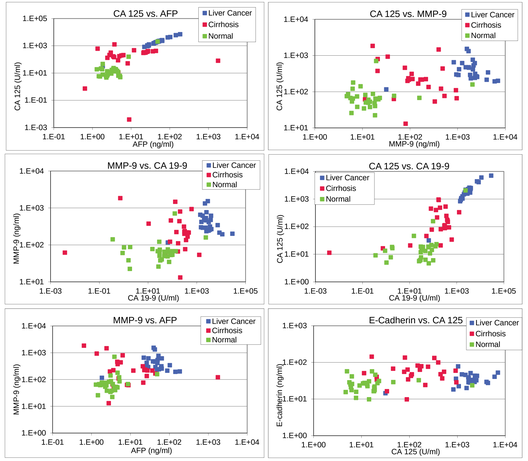


6 Performance of Paired Marker Combinations

Analysis of selected pairs of markers showed an improved ability to distinguish HCC samples from cirrhosis and normal samples as shown by the calculated assay sensitivities and specificities in Table 4 and in the 2-dimensional plots below. For the calculations of sensitivity and specificity, concentration cut-off values were selected for individual markers based on their ability to separate HCC cases from control samples. Both markers were required to be positive for HCC (i.e. above their respective cut-off values) to classify the sample as HCC. Table 4 demonstrates that even when this simple algorithm is used, specificity could be substantially increased by combining biomarkers results, with little or no cost to assay sensitivity. Combinations of these biomarkers should provide superior performance as compared to existing HCC detection modalities.

Biomarker (cut-off value)	HCC vs. Cirrhosis		HCC vs. Normal	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
AFP (>21 ng/ml)	96	72	96	97
CA 125 (>800 U/ml)	96	92	96	97
CA 19-9 (>1350 U/ml)	96	100	96	97
MMP9 (>190 ng/ml)	96	40	96	97
E-cadherin (<48 ng/ml)	88	60	88	7
AFP+CA 125	96	100	96	97
MMP9+CA 125	96	100	96	100
CA 19-9+MMP9	96	100	96	100
CA 125+E-cadherin	92	96	92	97
AFP+E-cadherin	84	96	84	97
AFP+MMP9	96	84	96	100

Table 4. Effect of combining results from pairs of biomarkers on the ability to detect HCC cases by differentiating them from controls. For individual biomarkers, samples were classified as HCC positive if they met the cut-off criteria listed next to the biomarker names. For pairs of biomarkers, samples were classified as HCC positive if the results for both individual biomarkers were HCC positive. Sensitivity is defined as the percent of HCC samples correctly classified as HCC. Specificity is defined as percent of non-HCC (normal or cirrhosis) samples correctly classified as non-HCC.



7 Conclusion

This preliminary study evaluates the levels of several classic cancer-associated markers in HCC patient sera and compares them to levels in sera from normal individuals and patients with benign liver disease (cirrhosis). The results show that CEA, CA 125, and CA 19-9 serum levels are significantly elevated in HCC patients as compared to cirrhotic and normal patients. CEA, CA 125, and CA 19-9 appear to be as effective as, if not superior to, AFP in diagnosing HCC and distinguishing HCC from cirrhosis in the small sample cohort tested. Furthermore, combining measurements from selected marker pairs distinguishes HCC from controls with a higher degree of specificity than is possible using individual markers. These observations must be corroborated by additional studies using larger, curated sample sets.

DOWNLOAD POSTER

Meso Scale Discovery®
A division of Meso Scale Diagnostics, LLC.
www.mesoscale.com®

130-CM-413