

Validation of Mass Spectrometric Serum Markers of Colorectal Cancer

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Abstract

Colorectal cancer (CRCa) is the third leading type of cancer in both men and women, with ~160,000 new cases and ~53,000 deaths expected to occur in the US in 2007. Over the last two decades, the incidence rate for colorectal cancer has decreased significantly, partially reflecting the appropriate screening of the disease. Unfortunately, many of the current screening methods are inundated with a variety of inadequacies such as poor sensitivity, poor specificity, expense, patient discomfort and limited application to screen patients at risk. Moreover, these methods are unable to detect early forms of CRCa. Therefore, diagnostic tools that are able to detect CRCa in its early stages can significantly improve survival rates. Previously, we reported the discovery of 27 biomarkers that were able to differentiate CRCa from non-CRCa patients using SELDI-TOF MS. When applied as a panel, these biomarkers were able to provide diagnostic tools of high sensitivity and specificity. In this study, validation of these biomarkers in an independent and geographically distinct set of samples was performed. Of the original 27 biomarkers (discovery set), six biomarkers validated and can be applied as a panel to a diagnostic algorithm giving ~98% sensitivity and ~14% specificity.

Introduction

CRCa is the third most common cancer diagnosed in North America, with approximately 160,000 new cases and ~53,000 deaths per year in the United States alone². Current screening methods include fecal occult blood tests, flexible sigmoidoscopy, colonoscopy, barium enema and computerized tomographic virtual colonoscopy^{3,4}. Currently, FOBT, FS and CS are recommended screening methods by the American Cancer Society, but suffer from a variety of inadequacies. These vary from one method to another, but include poor sensitivity, high numbers of false positives, patient discomfort, invasiveness and cost^{2,12}. The shortcomings are manifested at least in part through poor patient compliance with screening, with a recent survey indicating only one-third of people over 50 years having had an FS or CS within the previous five years.

Given the relative inadequacies of current colorectal cancer screening methods, there is a need for a simple and reliable (specific and sensitive) screening test to identify clinically relevant CRCa. The objective of this study was to (a) discover novel biomarkers in urine to differentiate CRCa from non-CRCa patients, and (b) to apply these to a preliminary diagnostic test.

Methods

Sample Collection. Blood serum samples for biomarker discovery were collected from patients recruited through several institutions and maintained by the European Tumor Sample Institute gGmbH. Additional samples for biomarker validation were obtained from the Fox Chase Cancer Center (Philadelphia, USA) (Table 1). Benign samples represent benign disease of the colon or rectum and control samples represent patients with no reported complaints of the colon or rectum.

Table 1. Distribution of samples for the discovery and validation of biomarkers for colorectal cancer.

Gender	Male		Female	
	ETSI	FCCC	ETSI	FCCC
CRCa	36	47	33	29
Benign	19	28	29	26
Control	9	45	14	28

ETSI: European Tumor Sample Institute
FCCC: Fox Chase Cancer Center
CRCa: Colorectal Cancer

Marker Validation: Samples were assayed as previously described¹ by SELDI-TOF MS using Q10 (quaternary amine chemistry) ProteinChip® arrays. In all, three sets of data were generated: ETSI samples assayed by themselves (biomarker discovery, previously reported¹); FCCC samples assayed by themselves (biomarker validation); and ETSI samples and FCCC samples assayed together (biomarker validation confirmation). Statistical comparisons were made for CRCa vs Non-CRCa, CRCa vs benign intestinal disease and CRCa vs healthy controls. A biomarker was considered to be potentially validated if it was statistically significant ($P < 0.05$, testing done using non-parametric Mann-Whitney rank sum tests) for at least one of the three comparisons and both the ETSI and FCCC samples in all three data sets (biomarker discovery, validation and validation confirmation). Validation was confirmed by manual review of mass spectral data to ensure that the biomarker was consistently up/down-regulated in CRCa compared to non-CRCa in all data sets for both ETSI and FCCC samples. The ROC-AUC for each validated biomarker in each dataset was calculated using the program JROCFit, and from these a mean ROC-AUC and associated standard deviation were calculated.

Diagnostic Test Development: Diagnostic tests using a panel of validated biomarkers were developed using proprietary methods. Spectra from FCCC samples assayed during biomarker validation confirmation were used as a training set of data. Spectra from ETSI samples assayed during biomarker validation confirmation were used as a test set of data.

Table 2. Summary of peaks capable of differentiating serum from healthy controls and/or benign colorectal disease patients from colorectal cancer patients.

Marker ID	ROC-AUC for the comparison of...		
	CRCa vs Benign	CRCa vs Ctrl	CRCa vs Non-CRCa
MCR-425	0.71 ± 0.14	-	0.62 ± 0.01
MCR-72C	0.66 ± 0.04	0.66 ± 0.03	0.65 ± 0.04
MCR-764	-	0.67 ± 0.05	-
MCR-2E4	0.64 ± 0.03	-	0.65 ± 0.02
MCR-D86	0.65 ± 0.04	0.65 ± 0.03	0.65 ± 0.02
MCR-5EF	-	0.66 ± 0.02	0.63 ± 0.04

CRCa: Colorectal Cancer
Benign: Benign disease
Ctrl: Healthy controls

Table 3. Summary of expression patterns for peaks capable of differentiating serum from healthy controls and/or benign colorectal disease patients from colorectal cancer patients. Expression patterns were consistent in both samples obtained from ETSI and from samples obtained from FCCC.

Marker ID	ETSI			FCCC		
	Mean	Median	SD	Mean	Median	SD
MCR-425	53.5	181.7	41.0	211.8	171.0	187.0
MCR-72C	15.1	21.2	13.6	20.0	19.5	23.6
MCR-764	28.9	50.4	26.2	37.5	37.3	41.5
MCR-2E4	6.7	4.3	2.5	3.9	3.0	1.6
MCR-D86	5.2	2.9	1.4	4.0	2.2	0.8
MCR-5EF	13.3	7.2	3.5	6.4	2.1	1.4

ETSI: European Tumor Sample Institute
FCCC: Fox Chase Cancer Center

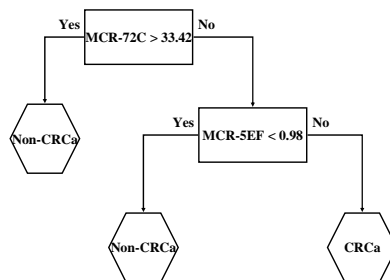


Figure 1. Classification methodology for CRCa based on validated serum biomarkers. A diagnostic model was derived using FCCC samples as a training set by selecting a peak intensity cutoff for a primary biomarker (MCR-72C) that gave a sensitivity of ~90% in the training sample population. Those patients on the side of this cutoff representing ~10% of all CRCa patients were given a non-CRCa diagnosis. A secondary biomarker (MCR-5EF) was then used to further classify the remaining patients in the training population to give the model depicted. The performance of this model was evaluated on a naive sample set obtained from FCCC.

Table 4. Evaluation of the sensitivity and specificity for diagnostic tests based on multiple serum biomarkers of colorectal cancer. Values given for sensitivity and specificity are expressed as percentages.

	FCCC Samples			ETSI Samples		
	MCR-72C	MCR-5EF	Combined	MCR-72C	MCR-5EF	Combined
Sensitivity	90.8	90.8	86.8	89.6	100	98.6
Specificity	20.3	55.4	43	9.9	7	14.1

ETSI: European Tumor Sample Institute
FCCC: Fox Chase Cancer Center

Results

Marker Validation. Statistical analysis of the spectra generated for this work indicated that six biomarkers capable of discriminating CRCa from non-CRCa patients in two sets of samples obtained from different institutions, and which were assayed on at least two different occasions (Table 2). The peaks listed were statistically significant ($P < 0.05$) in at least one of the three comparisons for each of four sets of samples assayed (ETSI biomarker discovery and validation confirmation, FCCC biomarker validation and validation confirmation). Values given are for the mean area under the receiver operator characteristic curve for the four sets of samples assayed. Error represents one standard deviation around the mean.

These markers fall in two general groups, those with amplified expression in CRCa compared to non-CRCa patients, and those with reduced expression in CRCa compared to non-CRCa patients (Table 3). The mean and median peak intensities shown in Table 3 for each biomarker peak, are also given in Table 2 in the format "CRCa - Ctrl", with the value for the group with higher expression in bold type. Differences between CRCa and non-CRCa patients were typically greater when looking at samples obtained from ETSI compared to samples obtained from FCCC.

Diagnostic Test Development. Derivation of a diagnostic algorithm was conducted using one biomarker from each of the two general groups outlined in Table 2, using spectra generated during biomarker validation confirmation from samples obtained from FCCC as a training dataset. These biomarkers were applied in a simple tree-type decision model to give a diagnosis of colorectal cancer or non-colorectal cancer (Figure 1), and performance was assessed on samples obtained from ETSI and assayed during biomarker validation confirmation (Table 4). Markers MCR-72C and MCR-5EF were used to generate a classification model using samples obtained from FCCC as a training data set. This model was then applied to the samples obtained from ETSI as a naive test data set.

Conclusions

This work describes the validation of serum biomarkers for colorectal cancer. These markers can be used to generate diagnostic algorithms applicable to groups of samples obtained from different sites with high sensitivity.

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Contributions and Acknowledgements

KS secured samples and medical history information for samples originating from ETSI, designed experiments and assisted in the preparation of arrays and in data evaluation. YZ prepared arrays, conducted mass spectrometric analysis. DSB secured samples and medical history information for samples originating from FCCC and conducted data analysis.

The authors would like to acknowledge the assistance in sample collection provided by the European Tumor Sample Institute gGmbH (ETSI-med, Hemmingdorf, Germany) and Fox Chase Cancer Centre (FCCC, Philadelphia, USA), in particular the efforts of Dr. Lader Fels (CEO, ETSI-med) and Dr. Andrew Godwin (Director, Biosample Repository, FCCC).

Materials. CiphergenExpress™ software, ProteinChip® arrays, CHCA, and PCS-4000 SELDI-TOF MS: Ciphergen Biosystems Inc. JROCFit software was accessed through <http://www.rad.jhmi.edu/javarad/roc/JROCFit.html> as needed. Miscellaneous chemicals: Sigma-Aldrich and Fisher.

