

Mass Spectrometric Serum Markers of Colorectal Cancer

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Abstract

Purpose: The purpose of this study was to discover biomarkers in serum that can differentiate patients with colorectal cancer (CRCa) from healthy controls and patients with benign colorectal disease. **Background:** CRCa is the third leading type of cancer, with ~150,000 new cases and ~56,000 deaths estimated in the US in 2005. Early detection of CRCa can significantly improve survival rates versus diagnosis at later disease stages. Current colorectal cancer screening tools suffer from a variety of shortcomings, which can include poor sensitivity, poor specificity, expense, patient discomfort and limited application to screen patients at risk. **Methods:** Proteomic screening of clinical serum samples by mass spectrometry was conducted to discover and characterize components of these samples for their ability to differentiate CRCa from non-CRCa patients. **Results:** 27 discrete proteins/peptides were discovered that could differentiate CRCa patient samples from healthy control and/or benign colorectal disease patient samples. Application of 5 of these serum components as biomarkers in a variety of single-model classification models with 10-fold cross validation showed sensitivity/specificity in excess of ~80%/80%. The use of 2 of these serum components with bagging meta-analysis and 10-fold cross validation gave sensitivity/specificity of ~85%/85%. **Conclusions:** The discovery of serum proteins and peptides with the potential to help detect and diagnose CRCa is presented.

Introduction

CRCa is the third most common cancer diagnosed in North America, with approximately 150,000 new cases and ~56,000 deaths per year in the United States alone¹. Current screening methods include fecal occult blood tests, flexible sigmoidoscopy, colonoscopy, barium enema and computerized tomographic virtual colonoscopy². Currently, FOBT, FS and CS are recommended screening methods by the American Cancer Society, but suffer from a variety of shortcomings. These vary from one method to another, but include poor sensitivity, high numbers of false positives, patient discomfort, invasiveness and cost³. 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^{4,5}.

Given the shortcomings 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 target proteins and peptides in urine to differentiate colorectal cancer from non-colorectal cancer patients, and (b) to apply these markers to a preliminary diagnostic test.

Methods

Sample Collection. Blood serum samples were collected from patients recruited through several institutions and maintained by the European Tumor Sample Institute gGmbH (Table 1). Contingency table analysis indicated no bias in patient diagnosis with regards to gender (> 0.165). A bias in patient age was identified, with ~12% of CRCa and ~38% of non-CRCa patients being under the age of 55 years. Upon receipt samples were thawed and divided into 25 µL aliquots for long-term storage at < -70°C. Available medical history information was transcribed into a relational database for ease of reference.

Table 1. Summary of the distribution of samples for the discovery of biomarkers for colorectal cancer. E: Ehrlanden, MD; Magdeburg.

Gender	Male			Female		
	E	MD	Total	E	MD	Total
CRCa	5	31	36	3	29	34
Benign	0	18	18	0	27	27
Healthy	0	9	9	0	14	14

Marker Discovery: Single 15 µL aliquots of each sample were thawed and combined with 60 µL of lysis solution (7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT and 2% (w/v) Ampholine) and incubated on ice for 15 minutes and then combined with 675 µL binding buffer (0.1 M Tris HCl pH 8.5). 300 µL of this mixture for each sample was incubated for 2 hours with vigorous mixing on duplicate spots on Q10 (quaternary amine chemistry) ProteinChip® arrays equilibrated with binding buffer. After incubation, each spot was washed twice, each time with 200 µL binding buffer for 15 minutes with vigorous shaking. Spots were then allowed to air dry prior to co-crystallization with sinapinic acid (SPA). Positive and negative controls were applied to each array to control for inter-array bias. Samples were assayed using a PCS4000 SELDI-TOF MS over a mass range of 0 to 30,000 m/z. Sample spectra were normalized for total ion current prior to automatic peak detection between 1,500 and 30,000 m/z. Peaks were retained only if detected in at least 10% of all spectra. Peaks from different spectra were paired and average peak intensities were compared between patient groups. The diagnostic utility of statistically significant peaks was evaluated using non-parametric statistical testing (Mann-Whitney rank sum tests) in conjunction with false discovery rate analyses, ROC-AUC statistics and attribute evaluation algorithms in the Waikato Environment for Knowledge Analysis (WEKA).

Diagnostic Test Development. Markers were used to generate diagnostic tests using several classification algorithms provided in WEKA. MCR-A42 and MCR-A61 were used separately and together with bagging meta-analysis based on the OneR algorithm, using a plurality of votes by ten different classifiers to derive a diagnosis. All diagnostic tests were created using 10-fold cross-validation.

Table 2. Summary of peaks capable of differentiating serum from healthy controls and/or benign colorectal disease patients from colorectal cancer patients. P values are given where P < 0.05. Values in parentheses indicate levels of false discovery rate (FDR) significance. Of those markers with FDR ≤ 1%, we expect to observe less than one that is falsely significant. Ctrl: Healthy controls. Benign: Benign colorectal disease.

Marker ID	Differentiates Colorectal Cancer from ...			Maximum ROC-AUC	Elevated in ...	
	Ctrl	Benign	Ctrl+Benign		CRCa	Non-CRCa
MCR-A61	3.31 x 10 ¹³ (1%)	6.04 x 10 ¹³ (1%)	2.51 x 10 ¹³ (1%)	0.95	-	√
MCR-A42	2.86 x 10 ¹³ (1%)	3.56 x 10 ¹² (1%)	5.83 x 10 ¹² (1%)	0.78	-	√
MCR-A6A3	8.72 x 10 ¹² (1%)	8.4 x 10 ¹² (1%)	2.15 x 10 ¹² (1%)	0.92	√	-
MCR-425	1.88 x 10 ¹³ (1%)	3.11 x 10 ¹³ (1%)	2.55 x 10 ¹³ (1%)	0.83	-	√
MCR-573	1.05 x 10 ¹³ (1%)	1.81 x 10 ¹³ (1%)	7.79 x 10 ¹² (1%)	0.83	√	-
MCR-CBE	5.44 x 10 ¹² (1%)	3.22 x 10 ¹³ (1%)	1.46 x 10 ¹³ (1%)	0.82	-	√
MCR-574	7.51 x 10 ¹² (1%)	1.37 x 10 ¹³ (1%)	7.26 x 10 ¹² (1%)	0.85	√	-
MCR-658	8.58 x 10 ¹² (1%)	1.65 x 10 ¹³ (1%)	2.66 x 10 ¹² (1%)	0.77	-	√
MCR-737	-	-	1.12 x 10 ¹³ (1%)	0.70	-	√
MCR-5B5	5.82 x 10 ¹² (1%)	3.60 x 10 ¹³ (1%)	1.82 x 10 ¹³ (1%)	0.72	-	√
MCR-7E7	1.19 x 10 ¹³ (1%)	3.67 x 10 ¹³ (1%)	2.81 x 10 ¹³ (1%)	0.71	-	√
MCR-6DF	1.15 x 10 ¹³ (1%)	2.26 x 10 ¹³ (1%)	1.37 x 10 ¹³ (1%)	0.71	-	√
MCR-AED	2.74 x 10 ¹² (1%)	-	6.51 x 10 ¹² (1%)	0.75	√	-
MCR-95C	2.00 x 10 ¹² (1%)	-	7.00 x 10 ¹² (1%)	0.75	√	-
MCR-85F	3.50 x 10 ¹² (1%)	-	1.15 x 10 ¹³ (1%)	0.74	√	-
MCR-3AD	1.67 x 10 ¹² (1%)	-	2.25 x 10 ¹² (1%)	0.75	√	-
MCR-3DB	2.38 x 10 ¹² (1%)	-	3.51 x 10 ¹² (1%)	0.74	√	-
MCR-031	-	6.58 x 10 ¹² (1%)	3.16 x 10 ¹² (1%)	0.63	√	-
MCR-764	0.039 (>10%)	7.41 x 10 ¹² (1%)	3.43 x 10 ¹² (1%)	0.69	-	√
MCR-FB1	-	1.54 x 10 ¹³ (1%)	7.26 x 10 ¹² (1%)	0.70	-	√
MCR-B25	-	0.036 (10%)	0.016 (10%)	0.62	√	-
MCR-300	0.032 (5%)	-	0.037 (>10%)	0.66	√	-
MCR-C9D	-	0.037 (5%)	0.045 (>10%)	0.62	-	√
MCR-0C3	9.28 x 10 ³ (10%)	-	0.046 (>10%)	0.68	-	√
MCR-04F	0.020 (10%)	-	0.037 (>10%)	0.67	-	√
MCR-523	-	0.019 (5%)	-	0.60	-	√
MCR-845	0.011 (10%)	-	-	0.66	-	√

Figure 1. Scatter-plot analyses of peak intensities of several colorectal cancer biomarkers and patient age. Biomarkers examined here include (A) MCR-A61, (B) MCR-6A3, (C) MCR-573, (D) MCR-425, (E) MCR-A42, and (F) MCR-CBE. Despite the apparent peak intensity increase with age for some biomarkers (for example, panel C), regression analysis using linear, exponential, power and logarithmic models did not identify significant correlations between peak intensity and age for any of these biomarkers. Red diamonds: CRCa samples. Black squares: non-CRCa samples (benign disease and controls).

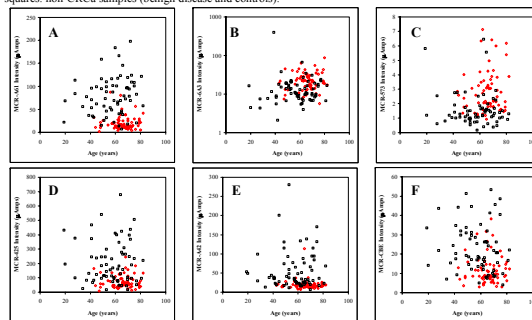


Table 3. Evaluation of the sensitivity and specificity for diagnostic tests based on multiple serum biomarkers of colorectal cancer. Markers MCR-A61, MCR-A42, MCR-425, MCR-573 and MCR-764 were used with 10-fold cross validation to generate a series of classification models. Error values give the 95% confidence interval of the mean for sensitivity and specificity. TP: true positive; TN: true negative; FP: false positive; FN: false negative.

Algorithm Used	Diagnosis Category...				Calculated...	
	TP	TN	FP	FN	Sensitivity (%)	Specificity (%)
J48 Tree	59	57	11	11	84.3 ± 8.5	83.8 ± 8.8
JRip	59	54	14	11	84.3 ± 8.5	79.4 ± 9.6
NBTree	56	55	13	14	80.0 ± 9.4	80.9 ± 9.4
OneR	57	57	11	13	81.4 ± 9.1	83.8 ± 8.8
PART	61	53	15	9	87.1 ± 7.8	77.9 ± 9.9
RiDoR	59	53	15	11	84.3 ± 8.5	77.9 ± 9.9

Table 4. Effects of bagging meta-analysis on sensitivity and specificity of two markers using the OneR algorithm. Diagnosis was based on the majority vote of 10 classification models, each generated using 10-fold cross validation. Error values give the 95% confidence interval of the mean for sensitivity and specificity.

Marker(s) Used	Sensitivity (%)	Specificity (%)	Correct (%)
MCR-A42 alone	88.6 ± 7.5	76.5 ± 10.1	82.6 ± 6.3
MCR-A61 alone	84.3 ± 8.5	85.3 ± 8.4	84.8 ± 6.0
MCR-A42 and MCR-A61	81.4 ± 9.1	82.4 ± 9.1	81.9 ± 6.4

Results

Marker Discovery. Statistical analysis of the spectra generated for this work indicated that several potentially useful markers for the diagnosis of colorectal cancer exist in the mass range studied. These include markers that can distinguish patients with benign disease from those with CRCa, control patients from those with CRCa, or non-CRCa patients from those with CRCa. Furthermore, there is overlap between the markers obtained for each of these indications (Table 2). These markers are unlikely to be the result of gender bias, but future studies will require the use of better age-matched controls to ensure that patient age is not a confounding factor. However, scatter-plot analysis of the six most significant biomarkers did not detect a correlation between signal intensity and patient age (Figure 1).

Diagnostic Test Development. Several different rule and tree-based algorithms were used to generate diagnostic tests with a selection of 5 of the 27 biomarkers discovered (MCR-A61, MCR-A42, MCR-425, MCR-573 and MCR-764, see Table 3). Application of one of these algorithms (OneR) to bagging meta-analysis of the two individually most significant markers (MCR-A42 and MCR-A61) using a majority vote of 10 different classifiers to generate a diagnostic decision improved test sensitivity and specificity, though not beyond the 95% confidence interval of the mean for either statistic (Table 4). A minimum of ten-fold cross-validation was used to promote test robustness.

Conclusions

This work describes the discovery of several serum biomarkers for colorectal cancer. These markers do not appear to be artefacts of patient gender, and the six most discriminatory markers do not appear to be artefacts of patient age. High sensitivity and specificity was observed in this sample set using a variety of classification algorithms. This was improved through bagging meta-analysis, but this improvement did not exceed the 95% confidence maxima for sensitivity and specificity generated by non-bagging analysis. These biomarkers have the potential to form the basis of diagnostic tools for the sensitive and specific diagnosis of colorectal cancer.

Quality assurance of the biomarkers described here using naive samples, as well as validation of the diagnostic tests developed, are currently underway.

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KS designed experiments and assisted in the preparation of arrays and in data evaluation. YZ prepared arrays, conducted mass spectrometric analysis, and assisted in experiment design and data evaluation. DSB secured samples and medical history information, conducted data analysis and assisted in data evaluation. The authors would like to acknowledge the assistance in sample collection provided by the European Tumor Sample Institute gGmbH (ETSI-med, Hennigsdorf, Germany), in particular the efforts of Dr. Luder Fels (CEO, ETSI-med).
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