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MAPRE1 as a plasma biomarker for early-stage colorectal cancer and adenomas

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Abstract

Blood-based biomarkers for early detection of colorectal cancer (CRC) could complement current approaches to CRC screening. We previously identified the APC-binding protein MAPRE1 as a potential CRC biomarker. Here we undertook a case-control validation study to determine the performance of MAPRE1 in detecting early CRC and colon adenoma and to assess the potential relevance of additional biomarker candidates. We analyzed plasma samples from 60 patients with adenomas, 30 with early CRC, 30 with advanced CRC, and 60 healthy controls. MAPRE1 and a set of 21 proteins with potential biomarker utility were assayed using high-density antibody arrays, and CEA was assayed using ELISA. The biologic significance of the candidate biomarkers

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was also assessed in CRC mouse models. Plasma MAPRE1 levels were significantly elevated in both patients with adenomas and patients with CRC compared with controls ($P < 0.0001$). MAPRE1 and CEA together yielded an area under the curve of 0.793 and a sensitivity of 0.400 at 95% specificity for differentiating early CRC from controls. Three other biomarkers (AK1, CLIC1, and SOD1) were significantly increased in both adenoma and early CRC patient plasma samples and in plasma from CRC mouse models at preclinical stages compared with controls. The combination of MAPRE1, CEA, and AK1 yielded sensitivities of 0.483 and 0.533 at 90% specificity and sensitivities of 0.350 and 0.467 at 95% specificity for differentiating adenoma and early CRC, respectively, from healthy controls. These findings suggest that MAPRE1 can contribute to the detection of early-stage CRC and adenomas together with other biomarkers.

Keywords

colorectal cancer; early detection; MAPRE1; blood-based biomarker; proteomics

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer death in both men and women in the United States (1). Most sporadic CRCs develop slowly over many years and often progress from early to advanced adenoma and then to invasive CRC (2). CRC is potentially curable if detected at an early stage; the 5-year survival rates for CRC are approximately 91% for localized disease but only about 13% if distant metastasis has occurred. Therefore, detecting adenoma and early-stage CRC is an attractive approach to reducing CRC mortality rates.

Colonoscopy is considered the gold standard for CRC screening owing to its ability to visualize the complete colon and to remove neoplastic lesions (3), but stool- or blood-based tests for CRC are more convenient, more cost effective, and less invasive than colonoscopy. Several clinical trials have reported that CRC screening with the fecal occult blood test reduced CRC-related mortality by approximately 16% (4). Although fecal occult blood tests have limited ability to detect adenomas, Imperiale et al. have recently reported that a stool DNA test combined with a fecal immunohistochemistry test provided higher sensitivity for detecting CRC and, to a lesser extent, advanced precancerous lesions (5).

Several potential blood-based biomarkers for early detection of CRC or for CRC risk assessment have been described (6-9). Carcinoembryonic antigen (CEA) is a circulating biomarker for CRC that is used in the clinical setting for monitoring therapy outcomes in patients with advanced disease and for predicting prognosis (10-12). However, CEA alone lacks the sensitivity and specificity to be used for early detection of CRC (12, 13). Additional biomarkers that complement CEA are needed for reliable and noninvasive detection of early-stage CRC.

We have previously undertaken a discovery study of potential circulating CRC biomarkers using mass spectrometry applied to pre-diagnostic samples from the Women's Health Initiative cohort, which resulted in the identification of several biomarker candidates (14). Prominent among the candidates was MAPRE1, which is known to bind APC (15, 16), a

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commonly mutated protein in CRC (17) and which plays a role in microtubule stabilization (18). The association between increased levels of circulating MAPRE1 and CRC was validated in independent plasma sample sets that consisted of newly diagnosed and pre-diagnostic CRC cases.

In the current study, we sought to determine the performance of MAPRE1 together with other candidate biomarkers for detecting disease in blood samples from patients with various stages of CRC or with adenoma collected under the auspices of the National Cancer Institute Early Detection Research Network.

MATERIALS AND METHODS

Human plasma samples

All human plasma samples were obtained following Institutional Review Board approval and informed consent. Plasma samples were collected through a consortium led by a Clinical Validation Center of the National Cancer Institute Early Detection Research Network at the University of Michigan. The samples were collected before any treatment at the time of diagnosis with adenoma (N = 60), early-stage CRC (N = 30; stage I [N = 11] and stage II [N = 19]), or late-stage CRC (N = 30; stage III [N = 21] and stage IV [N = 9]). Plasma samples from healthy controls were collected at the time of colonoscopy (N = 60) (Supplementary Table 1).

Mass spectrometry analysis of human plasma samples

Mass spectrometry analysis of human plasma samples was done as previously described (14, 19).

Mouse models and mass spectrometry analysis of mouse plasma samples

All animal experiments were conducted in accordance with institutional and national guidelines and regulations with approval by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center. Details on mouse models, plasma sample preparation, and mass spectrometry analysis of those samples are provided in the Supplementary Methods.

Human CRC cell lines and mass spectrometry analysis

A mass spectrometry analysis of CRC cell lines was performed as previously described (20). Eight CRC cell lines (HCT116, SW480, LoVo, SW620, HT29, SW48, Colo205, and Caco-2) were purchased from ATCC and grown in RPMI 1640 medium (Pierce) that contained 10% dialyzed fetal bovine serum (Invitrogen), 1% penicillin and streptomycin cocktail, and ^{13}C-lysine instead of regular lysine for seven passages, according to the standard SILAC protocol (21). Protein expression was estimated using normalized spectral counts (20).

High-density antibody array

In this study, we have expanded the validation of blood-based biomarkers for CRC and adenoma to include 22 candidates for which antibodies were available for assay using

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antibody microarrays (Supplementary Tables 2 and 3). High-density antibody array analysis was performed as previously described (22, 23). Briefly, albumin and IgG were depleted from plasma samples, 200 μg of the remaining plasma proteins from either cases or controls were labeled with Cy5, and a reference sample (a pool of plasma from seven healthy individuals) was labeled with Cy3. Array images were obtained using a GenePix 4000B microarray scanner (Molecular Devices), and the scanned array images were analyzed using GenePix Pro 6.0 image analysis software. The raw GenePix Array List file was aligned and resized to fit the individual spot features.

CEA enzyme-linked immunosorbent assay (ELISA)

Plasma levels of CEA were measured with an ELISA kit (R&D Systems) according to the manufacturer's protocol.

Histopathology analysis by tissue microarray

Tissue microarrays used in this study comprised 20 normal colonic tissues, 10 colon adenomas, and 66 CRCs (Folio Biosciences). The slides were deparaffinized, rehydrated, and antigen retrieval was performed using a pressure cooker with 0.01 M citrate buffer at pH 6.0. Intrinsic peroxidase activity was blocked using 1% hydrogen peroxide, and 10% normal goat serum (KPL) was used for 30 minutes to block non-specific antibody binding. The slides were then incubated overnight at 4°C with MAPRE1 antibody (1:200 dilution, Thermo Fisher Scientific). After incubation with the HRP-conjugated secondary antibody (Millipore) for 1 hour at room temperature, antigen signals were detected using the 2-Solution Diaminobenzidine Kit (Cell signaling) and counterstained with hematoxylin. The intensity of staining (0: negative, 1+: weakly positive, 2+: moderately positive, and 3+: strongly positive) and percentage of staining distribution in the tumor cells (from 0 to 100% of the cells) were evaluated for each TMA core. Two independent scoring was performed in a blind manner. Then the scoring was reviewed together again when discordant. In this study samples in which 10% or more of the cells with 1+ staining intensity were considered as positive.

Statistical analyses

For the high-density antibody array analysis, the change in the signal compared with the reference was calculated as $\log_2$. Technical sources of variation were normalized using Loess procedures, including within-array print-tip Loess and between-array quantile normalization. Following normalization, triplicate features were summarized using their median values. Individual biomarker performance was assessed using P values calculated by a one-sided Mann–Whitney U test, sensitivity, specificity, and the area under the curve (AUC) determined by a receiver operating characteristic analysis. The P values for significance of the biomarker panel combining MAPRE1 and CEA were subjected to a likelihood ratio test.

To develop an "OR" rule combination model, MAPRE1 and CEA were used as anchor biomarkers to develop a combination rule in which a patient was considered a case if any of the biomarkers exceeded a designated threshold or was ruled out as a case if all biomarkers were below those thresholds (24). The sensitivities at 90% specificity and at 95% specificity

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of the MAPRE1, CEA, and AK1 (“OR” rule combination model) were compared with those of MAPRE1 alone, CEA alone, and MAPRE1 plus CEA (linear combination). The P values for the differences in sensitivity were calculated by the McNemar test (25) and a bootstrap analysis repeated 1000 times. The differences were considered significant when P values were less than 0.05.

RESULTS

Plasma CEA levels were significantly higher in each CRC group than in healthy controls ($P = 0.0246$ for early CRC, $P < 0.0001$ for advanced CRC, and $P = 0.0002$ for total CRC, Mann–Whitney U test), but not significantly different between the adenoma and healthy control groups ($P = 0.4886$, Mann–Whitney U test) (Table 1). In contrast, the plasma levels of MAPRE1 were significantly elevated in both the adenoma and the CRC groups compared with healthy controls ($P < 0.0001$ for adenoma, $P = 0.0003$ for early CRC, $P = 0.0025$ for advanced CRC, and $P < 0.0001$ for total CRC, Mann–Whitney U test).

Protein expression of MAPRE1 was examined in colon adenoma and CRC tissues. Among 20 normal colonic tissues, 10 colon adenomas, and 66 CRCs in the tissue microarray, 9 (90.0%) colon adenomas and 45 (66.2%) CRCs were MAPRE1 positive while MAPRE1 expression was observed in only 5 (25.0%) normal colonic tissues (Supplementary Figure 1), suggesting that plasma MAPRE1 would be derived from colorectal tumors.

The combination of MAPRE1 and CEA yielded AUCs of 0.793 for early CRC and 0.731 for adenoma (Table 1 and Figure 1). The AUC of the two biomarkers together was significantly better than that of CEA alone ($P < 0.0001$ for adenoma, early CRC, and total CRC, and $P = 0.0014$ for advanced CRC, likelihood ratio test) or of MAPRE1 alone ($P = 0.0007$ for adenoma, $P = 0.0004$ for early CRC, $P = 0.0003$ for advanced CRC and $P < 0.0001$ for total CRC, likelihood ratio test). The combination of MAPRE1 and CEA yielded a sensitivity of 0.400 at 95% specificity in early CRCs compared with healthy controls, whereas the sensitivities of CEA alone and MAPRE1 alone at 95% specificity were noticeably lower for detecting early CRC**,** indicating an additive effect of the combination of MAPRE1 and CEA for detecting early CRC.

We determined the individual performances of biomarker candidates for which antibodies were printed on the microarrays (Table 2). Three proteins (AK1, CLIC1, and SOD1) were significantly elevated in adenoma and in each CRC group compared with healthy controls ($P < 0.05$, Mann–Whitney U test). Five additional proteins (AZGP1, CALR, LGALS3BP, SPARC, and ZYX) exhibited significantly elevated levels in at least one disease group compared with healthy controls. AK1, CLIC1, and SOD1 had been previously identified in a proteomic analysis of pre-diagnostic plasma samples from the Women’s Health Initiative cohort (Supplementary Table 2). Mass spectrometry analysis yielded substantial peptide coverage for AK1, CLIC1, and SOD1 (Figure 2), indicating the occurrence of full-length proteins for these three biomarkers in human plasma. To determine whether these proteins originated from tumor cells, we performed proteomic analysis of three different compartments (media, surface, and whole-cell extract) of eight CRC cell lines. CLIC1 and

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SOD1 were broadly expressed in the media of CRC cell lines (Supplementary Table 4), while AK1 was identified in the media of three CRC cell lines.

To further investigate the association of increased circulating levels of these biomarker candidates with early CRC development, we used mass spectrometry to profile plasma samples collected at different time points during tumor development from two genetically engineered CRC mouse models. Levels of AK1 and SOD1 were elevated in plasma samples from Apc- and Msh2-deficient mice (26) with low-grade and high-grade adenomas compared with controls (Figure 3). CLIC1 levels were elevated at pre-clinical timepoints (at 2 weeks and 4 weeks after induction of $Kras^{G12D}$) in a mouse with oncogenic Kras-induced CRC compared with controls (unpublished mouse model) (Figure 3). These findings further support a biologic basis for the association between increased plasma levels of these three biomarker candidates and early development of CRC.

Since CEA was not significantly elevated in adenoma but was significantly elevated in CRCs at all stages, we investigated whether an “OR” rule (24), rather than a linear combination rule, better discriminates between cases (adenomas or CRCs) and controls. To rule in an asymptomatic person for subsequent work-up (i.e., colonoscopy) who otherwise does not plan to take part in screening, a biomarker test with sensitivity at 90% or with 95% specificity would have potential clinical utility. As reported in Table 1, the combination of MAPRE1 and CEA using logistic regression yielded a sensitivity of 0.400 at 95% specificity in a comparison of early CRC and healthy controls and a sensitivity of only 0.167 at 95% specificity in a comparison of adenoma and healthy controls. Therefore, we assessed the potential of an “OR” rule strategy in which any biomarker in the panel exceeding a certain threshold identifies a patient as a case and in which all biomarkers in the panel not exceeding their designated thresholds would rule out a patient as a case. The “OR” rule combination of MAPRE1, CEA, and AK1 yielded significantly higher sensitivity at 95% specificity than the combination of MAPRE1 and CEA in a comparison of adenoma with controls (0.167 sensitivity for MAPRE1 and CEA and 0.350 sensitivity for the “OR” rule combination of MAPRE1, CEA, and AK1; P = 0.015, McNemar test; P = 0.099, bootstrap) and in a comparison of advanced CRC with controls (0.233 sensitivity for MAPRE1 and CEA and 0.500 sensitivity for the “OR” rule combination of MAPRE1, CEA, and AK1; P = 0.013, McNemar test; P = 0.071, bootstrap) (Table 3). For early CRC compared with controls, the sensitivity at 95% specificity of the “OR” rule combination of MAPRE1, CEA, and AK1 (0.467) was higher than that of MAPRE1 and CEA (0.400); however, the difference between these sensitivities did not reach statistical significance (Table 3). The sensitivities of the “OR” rule combination of MAPRE1, CEA, and AK1 at 90% specificity were 0.483 for adenoma, 0.533 for early CRC, and 0.633 for advanced CRC, and the sensitivities of MAPRE1 and CEA at 90% specificity were 0.400 for adenoma, 0.433 for early CRC, and 0.400 for advanced CRC. These findings suggest an incremental increase in diagnostic performance due to integrating AK1 as a biomarker with MAPRE1 and CEA in screening for adenoma.

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DISCUSSION

Our study focused on testing the merits of MAPRE1 as a circulating biomarker for CRC and adenomas, both alone and in combination with CEA. We also explored the potential contributions of additional candidate biomarkers. Our prior finding of increased levels of MAPRE1 in pre-diagnostic CRC plasmas (14), which led to the present study, is of particular interest, considering the biologic significance of this protein in the context of CRC (15-17). Concordant with the results of immunohistochemical staining of MAPRE1 in this study, recent studies have demonstrated that MAPRE1 is upregulated in pre-malignant colon mucosa of a rat model of chemically induced CRC and an APC-mutant rat model (27), suggesting that MAPRE1 expression is associated with early field carcinogenesis of CRC. Overexpression of MAPRE1 also has been associated with poor prognosis in CRC (28). In this study, we validated that plasma MAPRE1 levels are significantly associated with CRC and adenoma using an independent sample set from the University of Michigan. MAPRE1 with and without CEA was significantly associated with early CRC and adenoma.

Currently, the repertoire of biomarkers with demonstrated ability to detect adenoma is quite limited. In a recent validation study of a multitarget stool DNA test for CRC screening, the sensitivity for advanced precancerous lesions was 42.4% with DNA testing and 23.8% with a fecal immunochemical test. The rate of detection of polyps with high-grade dysplasia was 69.2% with DNA testing and 46.2% with the fecal immunochemical test (5). Plasma methylated SEPT9 has been shown to be a biomarker for CRC in a screening setting, with a sensitivity of 48.2% and a specificity of 91.5%, but its sensitivity for advanced adenoma was only 11.2% (6). For MAPRE1 and CEA, the sensitivity at 90% specificity was 40% for adenoma and 43% for early CRC (Table 3), suggesting that MAPRE1 combined with CEA may improve current blood-based screening for both adenoma and CRC. Further studies will be warranted to validate the performance of MAPRE1 together with CEA, particularly in high-risk adenoma (villous histology, high-grade dysplasia, size larger than 1 cm, or 3 or more adenomas).

We also determined the individual performances of additional biomarker candidates. Eight candidates were significantly associated with disease in one or more groups, of which three candidates (AK1, CLIC1, and SOD1) were each significantly associated with adenoma, early-stage CRC, advanced-stage CRC, and total CRC. These three candidates were previously identified in a proteomic analysis of pre-diagnostic plasma samples from the Women's Health Initiative cohort (Supplementary Table 2).

Evidence also suggests that a systemic increase in circulating AK1 may occur very early in the development of colorectal neoplasia. We found that plasma levels of AK1 were significantly higher in mice with low-grade as well as those with high-grade adenoma compared with controls and that AK1 was elevated in plasma samples from human adenoma and CRC cases compared with controls. Recent studies have indicated a role of AK1 in the bloodstream in regulating metabolism of extracellular AMP, ADP, and ATP (29), which are associated with immunosuppression and tumor progression (30). Expression of AK1 increases in response to obesity and metabolic syndrome, which are known risk factors of CRC (31-33).

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We also found increases in plasma levels of CLIC1 in preclinical CRC and SOD1 in adenomas compared with controls in mouse experiments, and those biomarkers were also elevated in plasma samples from human adenoma and CRC cases compared with controls. CLIC1 is a previously characterized plasma biomarker for nasopharyngeal carcinoma and has been found to promote cell migration and invasion in CRC (34-37). SOD1 protein expression in CRC tumors and precancerous colon tissues from chemically induced CRC mouse or rat models has been investigated, with variable results (34, 38-41).

Although the “OR” rule combination of MAPRE1, CEA, and AK1 yielded the highest sensitivity in this study compared with the Lasso logistic regression model and two other machine learning methods (optimal linear combination by maximizing the partial area under the receiver operating characteristic curve and combination of biomarkers using fractional polynomials) (data not shown), the merits of the “OR” rule will need to be assessed through further testing in additional, larger sample sets. The bootstrap test used the decision rule applied to subjects not included in the bootstrap sample; thus, the test does not have the bias associated with estimating performance using the same sample set used for training.

Our previous discovery studies and our validation studies presented here stem from an in-depth quantitative proteome analysis of plasma that addresses the intended application of blood-based screening for adenoma and early CRC in combination with other screening modalities. Plasma contains a rich assortment of circulating molecules and cellular materials that can inform us about tumor development, including tumor-derived DNA (6, 42), non-coding RNAs (43, 44), autoantibodies (45, 46), and metabolites (47). There remains a pressing need to determine the relevance and relative contributions of the various potential biomarkers in detecting adenoma and early CRC. Our validation of MAPRE1 as a circulating biomarker for adenoma and CRC provides a rationale for further studies into the development of a blood-based biomarker approach using MAPRE1 that complements current screening modalities for CRC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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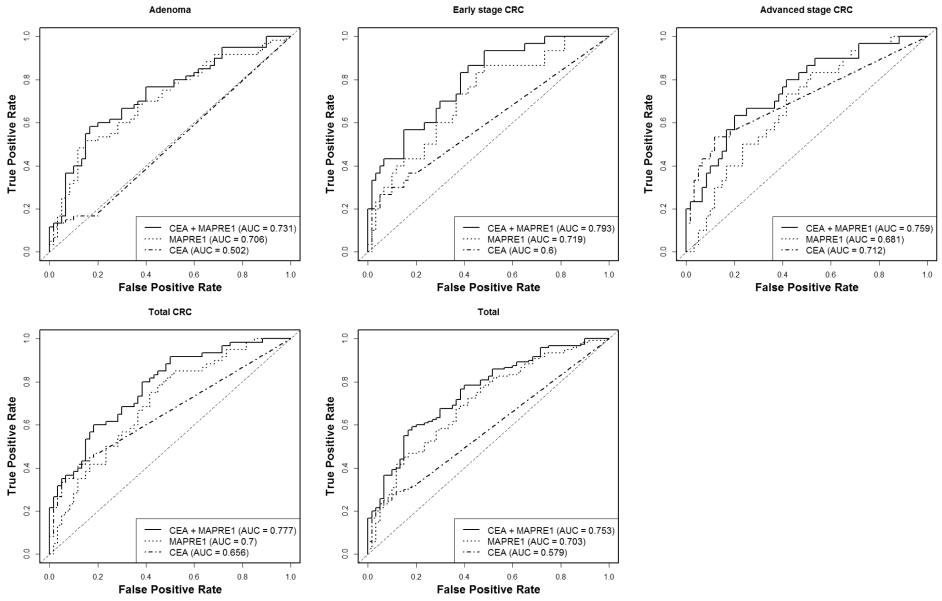

Figure 1. Performance of CEA, MAPRE1, and CEA combined with MAPRE1 in the University of Michigan sample set

Receiver operating characteristic curves for CEA, MAPRE1, and the combined panel of CEA and MAPRE1 in the comparison of adenoma, early-stage CRC, advanced-stage CRC, total CRC, and total cases with healthy controls.

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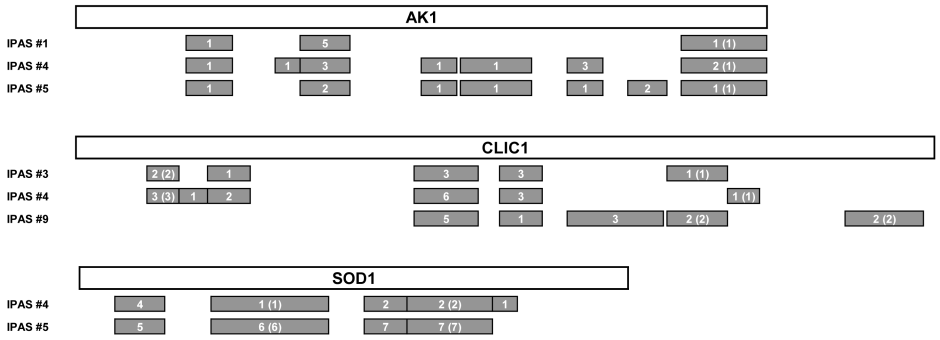

Figure 2. Mass spectrometry identification of biomarker candidates in the pre-diagnostic plasmas in the Women's Health Initiative cohort

Schema of three biomarker candidates (AK1, CLIC1, and SOD1) and identification of peptides by mass spectrometry. Gray bars indicate identified peptides. Numbers indicate mass spectra counts for each peptide, and numbers in parentheses indicate mass spectra counts with quantification. The amino acid sequence is based on P00568-1 for AK1, O00299-1 for CLIC1, and P00441-1 for SOD1 in the UniProt Knowledgebase.

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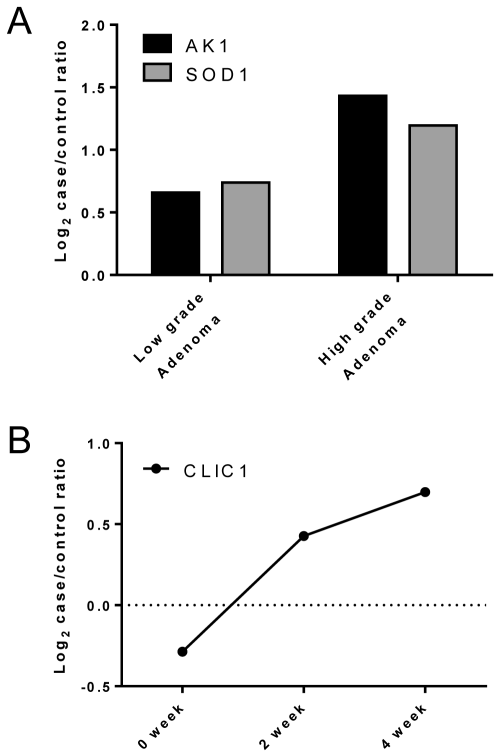

Figure 3. Quantification of biomarker candidates in pre-diagnostic plasmas from mouse models of CRC

A. Log_2-transformed case/control ratios of AK1 and SOD1 levels in plasma from *Apc*- and *Msh2*-deficient mice with low-grade adenoma and high-grade adenoma compared with controls. B. Log_2-transformed case/control ratios of CLIC1 levels in plasma from a mouse with oncogenic $Kras^{G12D}$-induced CRC compared with controls. Plasma samples were collected at 0 weeks, 2 weeks, and 4 weeks (the mice were sacrificed at 5 weeks) after induction of $Kras^{G12D}$ from mice in which colon adenocarcinoma developed, and those samples were compared with a pool of plasma samples from five sex-matched mice without tumors.

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Table 1

Performance of CEA and MAPRE1 in the University of Michigan sample set.

Marker (Supplier)	Parameter	Adenoma (N = 60) vs. healthy controls (N = 60)	Early CRC (N = 30) vs. healthy controls (N = 60)	Advanced CRC (N = 30) vs. healthy controls (N = 60)	Total CRC (N = 60) vs. healthy controls (N = 60)	Total cases (N = 120) vs. healthy controls (N = 60)
CEA (ELISA; R&D Systems)	P value	0.4886	0.0246	<0.0001	0.0002	0.0422
	AUC (95% CI)	0.502 (0.430-0.574)	0.600 (0.494-0.706)	0.712 (0.605-0.820)	0.656 (0.574-0.738)	0.579 (0.515-0.643)
	Sensitivity at 95% specificity	0.133	0.267	0.400	0.333	0.233
MAPRE1 (Strategic Diagnostics Inc.)	P value	<0.0001	0.0003	0.0025	<0.0001	<0.0001
	AUC (95% CI)	0.706 (0.612-0.799)	0.719 (0.608-0.830)	0.681 (0.568-0.793)	0.700 (0.606-0.793)	0.703 (0.622-0.784)
	Sensitivity at 95% specificity	0.250	0.267	0.100	0.183	0.217
MAPRE1 + CEA	AUC	0.731	0.793	0.759	0.777	0.753
	P value (vs. CEA)	<0.0001	<0.0001	0.0014	<0.0001	<0.0001
	P value (vs. MAPRE1)	0.0007	0.0004	0.0003	<0.0001	<0.0001
	Sensitivity at 95% specificity	0.167	0.400	0.233	0.350	0.258

CI: confidence interval

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Table 2

Biomarker candidates significantly elevated in the University of Michigan sample set.

Marker (Supplier)	Parameter	Adenoma (N = 60) vs. healthy controls (N = 60)	Early- CRC (N = 30) vs. healthy controls (N = 60)	Advanced- CRC (N = 30) vs. healthy controls (N = 60)	Total CRC (N = 60) vs. healthy controls (N = 60)	Total cases (N = 120) vs. healthy controls (N = 60)
AK1 (Strategic Diagnostics, Inc.)	P value	0.0021	0.0001	0.0002	<0.0001	<0.0001
	AUC	0.652	0.755	0.732	0.744	0.698
AZGP1 (MyBioSource)	P value	0.4572	0.2069	0.0233	0.0424	0.1442
	AUC	0.506	0.553	0.629	0.591	0.549
CALR (Aviva Systems Biology)	P value	0.1512	0.0178	0.0734	0.0146	0.0314
	AUC	0.555	0.637	0.594	0.616	0.585
CLIC1 (Strategic Diagnostics, Inc.)	P value	0.0180	0.0121	0.0036	0.0012	0.0015
	AUC	0.611	0.647	0.675	0.661	0.636
LGALS3BP (Aviva Systems Biology)	P value	0.2353	0.0455	0.4710	0.1613	0.1606
	AUC	0.538	0.610	0.495	0.553	0.545
SOD1 (Strategic Diagnostics, Inc.)	P value	0.0424	0.0279	0.0126	0.0054	0.0067
	AUC	0.591	0.624	0.646	0.635	0.613
SPARC (Santa Cruz Biotechnology)	P value	0.2500	0.0416	0.1433	0.0428	0.0828
	AUC	0.536	0.613	0.569	0.591	0.564
ZYX (Sigma-Aldrich)	P value	0.0241	0.2647	0.0602	0.0900	0.0274
	AUC	0.605	0.541	0.601	0.571	0.588

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Table 3

Sensitivities at 90% and 95% specificity of biomarkers and their combinations.

Parameter	Model		Adenoma	Early-CRC	Advanced CRC	Total CRC	Total case
Sensitivity at 95% specificity	**MAPRE1+CEA+AK1 (“OR” rule)**		**0.350**	**0.467**	**0.500**	**0.433**	**0.375**
	P value (McNemar test, Bootstrap)	vs. MAPRE1+CEA	0.015, 0.099	0.617, 0.308	0.013, 0.071	0.228, 0.037	0.008, 0.042
		vs. MAPRE1	0.041, 0.007	0.077, 0.005	0.003, <0.001	0.001, <0.001	0.002, <0.001
		vs. CEA	0.012, <0.001	0.077, 0.005	0.450, 0.061	0.239, 0.001	0.004, <0.001
	MAPRE1+CEA		**0.167**	**0.400**	**0.233**	**0.350**	**0.258**
	MAPRE1		**0.250**	**0.267**	**0.100**	**0.183**	**0.217**
	CEA		**0.133**	**0.267**	**0.400**	**0.333**	**0.233**
Sensitivity at 90% specificity	**MAPRE1+CEA+AK1 (“OR” rule)**		**0.483**	**0.533**	**0.633**	**0.567**	**0.500**
	P value (McNemar test, Bootstrap)	vs. MAPRE1+CEA	0.182, 0.075	0.248, 0.067	0.023, 0.020	0.003, 0.008	0.006, 0.009
		vs. MAPRE1	0.016, 0.016	0.077, 0.006	0.002, 0.002	<0.001, <0.001	<0.001, <0.001
		vs. CEA	<0.001, <0.001	0.013, 0.001	0.074, 0.011	0.003, <0.001	<0.001, <0.001
	MAPRE1+CEA		**0.400**	**0.433**	**0.400**	**0.383**	**0.392**
	MAPRE1		**0.333**	**0.367**	**0.200**	**0.283**	**0.308**
	CEA		**0.167**	**0.300**	**0.467**	**0.383**	**0.275**

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