Increased plasma levels of the APC-interacting protein MAPRE1, LRG1 and IGFBP2 preceding a diagnosis of colorectal cancer in women

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Abstract
Longitudinal blood collections from cohort studies provide the means to search for proteins associated with disease prior to clinical diagnosis. We investigated plasma samples from the Women’s Health Initiative (WHI) cohort to determine quantitative differences in plasma proteins between subjects subsequently diagnosed with colorectal cancer (CRC) and matched controls that remained cancer free during the period of follow-up. Proteomic analysis of WHI samples collected prior to diagnosis of CRC resulted in the identification of six proteins with significantly \((p < 0.05)\) elevated concentrations in cases compared to controls. Proteomic analysis of two colorectal cancer cell lines showed 5 of the 6 proteins were produced by cancer cells. MAPRE1, IGFBP2, LRG1 and CEA were individually assayed by enzyme linked immunosorbent assay (ELISA) in 58 pairs of newly diagnosed CRC samples and controls and yielded significant elevations \((p < 0.05)\) among cases relative to controls. A combination of these four markers resulted in an ROC with an AUC=0.841 and 57% sensitivity at 95% specificity. This combination rule was tested in an independent set of WHI samples collected within 7 months prior to diagnosis from cases and matched controls resulting in 41% sensitivity at 95% specificity. A panel consisting of CEA, MAPRE1, IGFBP2 and LRG1 has predictive value in pre-diagnostic colorectal cancer plasmas.

Keywords: colorectal cancer, risk markers, Pre-Diagnostic samples

Abbreviations:

ARMET, Mesencephalic astrocyte-derived neurotrophic factor
CEA, carcinoembryonic antigen
ENO1, alpha enolase
IGFBP2, insulin-like growth factor binding protein 2
LRG1, leucine-rich alpha-2-glycoprotein  
MAPRE1, microtubule-associated protein RP/EB family member 1  
PDIA3, protein disulfide-isomerase A3  
ELISA, enzyme-linked immunosorbent assays  
IPAS, intact protein analysis system  
SILAC, standard stable isotope labeling with amino acids in cell culture

**Introduction**

Current screening methods for colorectal cancer have had an impact on mortality associated with this disease(1). There is a 30-40% drop in risk of developing CRC following a negative result from a colonoscopy and as much as a 50% reduction in incidence in the portion of the bowel examined by sigmoidoscopy or colonoscopy(2). Even with these decreases in risk and incidence, it is estimated that ~60% of subjects over the age of 50 in the United States are not screened at recommended intervals(3, 4). In the case of colonoscopies, even when subjects are referred by their physician there is only a ~50% rate of adherence(5).

Plasma levels of CEA are currently utilized as a preoperative prognostic indicator for CRC, with higher levels of CEA positively correlated with a poor prognosis(6). CEA also has utility for monitoring therapy in advanced disease and for patient surveillance following curative resection(7, 8). However it lacks the sensitivity and specificity to be used as a diagnostic marker for CRC(6), hence the need for additional markers that could supplant it or augment its performance.

The ease of sampling plasma makes it a logical choice for the development of a panel of proteins that inform about risk of developing CRC. However, the plasma proteome is extremely complex and is composed of proteins ranging in concentration over at least 9 orders of magnitude. Recent work has shown that low-abundance plasma proteins may
be identified with high confidence following extensive plasma fractionation(9). High abundant proteins interfere with detection and quantification of less abundant proteins, necessitating their removal prior to MS analysis, typically through immunodepletion. After removal of the most highly abundant proteins, samples still require extensive fractionation by anion exchange and/or reverse-phase chromatography to decomplex the sample to achieve adequate sampling of the plasma proteome.

Guidelines for the design of biomarker discovery and validation studies have been recommended(10). Retrospective longitudinal repository studies are used to evaluate biomarkers for their capacity to detect preclinical disease as a function of time before clinical diagnosis, as well other sample characteristics that may define clinical applications. This is done through analysis of the most promising markers and developing algorithms for screening positivity based on a combination of markers. The use of specimens collected prior to diagnosis through longitudinal cohort studies meets prospective-specimen-collection, retrospective-blinded-evaluation (PRoBE) design requirements(11), reduces bias and allows identification of proteins that may have value for early detection and risk assessment. Using samples from the WHI cohort, an intact protein analysis system (IPAS) approach that allows quantitative analysis of proteins over six to seven orders of magnitude of abundance(9, 12-14) was applied to plasmas from 90 participants who were subsequently diagnosed with colon cancer within 18 months following blood draw and to 90 matched controls from the same cohort. Further testing of a protein subset was performed in samples from Early Detection Research Network (EDRN) collected at the time of diagnosis which included both male and female subjects. A panel established in the newly diagnosed cohort was subsequently shown to have
predictive value in an independent set of pre-diagnostic colorectal cancer plasmas from the WHI cohort.

**Methods**

**Study Population**

The sample population used in the discovery phase consisted of plasmas from 90 women who were diagnosed with colorectal cancer within 18 months following a blood draw that occurred in the 3\textsuperscript{rd} year of participation in the WHI Observational Study. These cases were individually matched based on age (± 2 years), race/ethnicity, and baseline blood draw (± 2 months) to a randomly selected control without a history of cancer diagnosis (Table 1).

Plasma from 58 newly diagnosed male and female patients with colorectal cancer and 58 matched controls were collected through the Community Clinical Oncology Program at the University of Michigan, Ann Arbor, MI, following informed consent. Cases were individually matched based on age (within 3 years) and gender.

An independent set of plasmas from 32 subjects in the WHI Observational Study that were diagnosed with CRC within 7 months following the 3\textsuperscript{rd} year blood draw and 32 matched controls was used for validation. Matching was done based on age (± 4 years), race/ethnicity, baseline blood draw (± 4 months), BMI, hormone therapy use and a negative history for cancer (Table 1).
Proteomic Analysis

*Intact Protein Analysis System (IPAS)*

Nine large scale proteomic experiments were performed on pools of plasmas from 10 cases and 10 controls as previously described (12, 15) (Supplementary Figure 1). In four experiments, the pool of cases was labeled with light acrylamide, and its matched control pool was labeled with heavy 1,2,3-\textsuperscript{13}C-acrylamide isotope. The labeling was switched in the other case/control pools. In each experiment, the pool of cases and the pool of matched controls were mixed together prior to further processing and mass spectrometry.

Proteins were separated by an automated online 2D-HPLC system controlled by Workstation Class-VP 7.4 (Shimadzu Corporation). Separation consisted of anion exchange chromatography followed by reversed-phase chromatography. In-solution tryptic digestion was performed with lyophilized aliquots from the reversed-phase (second dimension) fractionation step. Aliquots were subjected to mass spectrometry shotgun analysis using an LTQ-Orbitrap (Thermo) mass spectrometer coupled with a NanoLC-1D (Eksigent). The acquired data were automatically processed by the Computational Proteomics Analysis System (CPAS)(16). For the identification of proteins with false positive error rate <5%, LC-MS/MS spectra of the samples were subjected to tryptic searches against the human IPI database (v.3.13) using X!Tandem(17). Search results were then analyzed by PeptideProphet(18) and ProteinProphet(19) programs. Quantitative protein analysis was based on differential labeling of cysteine residues with acrylamide isotopes. Peptide isotopic ratios were plotted in logarithmic scale in a histogram and the median of the distribution was
centered at zero (Supplementary Figure 2). All normalized peptide ratios for a specific protein were averaged to compute an overall protein ratio. Reported statistical significance of the protein quantitative information was obtained using a one-sample t-test. False Discovery Rates (FDR) were calculated based on the distribution of p-values from permutations of disease labels and the observed p-values from the original data.

**Colorectal cancer cell line proteomic analysis**

HCT116 and SW480 were prepared according to the standard stable isotope labeling with amino acids in cell culture (SILAC) protocol as previously described(20). Secreted proteins were obtained directly from conditioned media after 48h of culture. Total cell extract (TCE) was obtained by sonicating ~2x10^7 cells followed by centrifugation at 20,000g. A surface-enriched fraction was obtained by biotinylating ~2x10^8 cells in culture plates. Proteins were extracted in a 2% NP40 solution and subsequently isolated using neutravidin.

Cell line preparations were fractioned by reversed phase chromatography. Reversed phase fractions from each preparation were individually digested with trypsin and grouped into 23-27 pools based on chromatographic features. LC-MS/MS and protein identification were performed as described above using v3.57 of the human IPI database.

**ELISA-based validation**

Human IGFBP2 (R&D Systems), LRG1 (IBL-America), CEA (Genway Biotech, Inc.), and MAPRE1 (USCN Life) measurements were performed on newly diagnosed and pre-diagnostic plasma samples according to the manufacturer’s protocol. Absorbance was measured using a SpectraMax Plus 384 and results calculated with SoftMax Pro v4.7.1 (Molecular Devices). p-values were computed using a paired Mann-Whitney Wilcoxon
test on raw concentration values. ELISA measurements above and below the detection limit for assays were imputed by the maximum and minimum computable values for the assay.

Results

Proteomic analysis of plasma from study subjects and CRC cell lines

An in-depth quantitative MS analysis of WHI plasma samples in 9 large scale experiments yielded a total of 1,992,567 mass spectra, resulting in a total of 5,022 unique protein IDs in the International Protein Index (IPI) database(21). Quantitative data based on isotopic ratios for case vs control was obtained for 1,779 proteins. An overall p-value and a geometric mean ratio for each protein across all 9 experiments were calculated. Six proteins were significantly (p<0.05) elevated in cases compared to controls with a case-to-control ratio >1.2 (Table 2). MAPRE1 is a cytoplasmic protein that binds to adenomatous polyposis coli (APC), a commonly mutated gene in colorectal adenocarcinoma, and that functions in mitotic processes(22). LRG1 is an extracellular protein whose function is largely unknown with varied expression levels in tissues(23). A role for LRG1 in granulocyte differentiation has been suggested(24). IGFBP2 is an extracellular protein that binds IGF2 and has been shown to potentially have both proliferative and anti-proliferative roles in cancer(25). Enolase 1 has been identified as a central element in a disease-specific gene network in colon cancer(26). ARMET and PDIA3 belong to a family of endoplasmic reticulum stress induced proteins which have been found to be upregulated in gastric and hepatocellular carcinomas(27, 28).
spectrometry analysis yielded substantial peptide coverage for all six proteins (Figure 1a-1f), indicating a robust identification of each full-length protein in human plasma.

To determine if the identified proteins may have originated from tumor cells or from a host response, proteomic analysis of two CRC cell lines with different driver mutations was performed using SILAC(20). HCT116 and SW480 were analyzed to assess potential differences in protein expression based on APC mutational status. MAPRE1, IGFBP2, ENO1, PDIA3 and ARMET were identified in both HCT116 and the APC-mutant SW480 (Supplementary Figure 3a-3e), while LRG1 was not identified in either of the two cell lines. MAPRE1, ENO1, PDIA3 and ARMET were observed in total cell extracts (TCE), the media and surface-enriched fractions in both cell lines. The APC-binding domain of MAPRE1 was enriched in the media and cell surface fractions. PDIA3 was enriched in the cell surface compartment with fewer peptides identified in the conditioned media. ARMET was also enriched on the cell surface of the SW480 cell line, but not in HCT116. ENO1 was the most identified protein in both TCE and conditioned media. IGFBP2 was predominantly observed in the conditioned media, with few peptides identified in the TCE. These cell findings suggest that tumor cells may contribute to increased levels observed in plasma for MAPRE1, IGFBP2, ENO1, PDIA3 and ARMET.

**Assays of IGFBP2, LRG1 and MAPRE1 in plasmas from newly diagnosed CRC cases.**
Three of these six proteins (MAPRE1, LRG1 and IGFBP2) had ELISA assays available for further validation studies. IGFBP2, LRG1 and MAPRE1 along with CEA were assayed in plasma from newly diagnosed CRC subjects and controls (Figure 2). Given that the discovery studies were based on pools of cases and controls, ELISA assays of individual samples were relied upon to develop a combination rule for validation of the marker panel in an independent set of pre-diagnostic samples. All four of the assayed proteins were found to be significantly (p <0.05) elevated >1.5-fold in cases compared to controls (Table 3) in a set of 58 newly diagnosed CRC cases and 58 age-matched controls. AUCs for IGFBP2, LRG1, MAPRE1 and CEA ranged from 0.712 to 0.782 (Table 3). Linear regression analyses based on maximum likelihood estimation of raw ELISA values were performed on all possible combinations of the four markers. A combination of all four markers (denoted “Panel”) was found to have the highest AUC of 0.841 with 59% sensitivity at 95% specificity, a 23% increase over CEA alone (Figure 3a). Scatter plots of ELISA responses showed levels of MAPRE1 and CEA correlated well (Supplementary Figure 4). Similarly, IGFBP2 and LRG1 were also highly correlated, while MAPRE1 and LRG1 exhibited an orthogonal relationship.

**Assays of individual markers in an independent set of pre-diagnostic plasmas**

The linear combination of CEA, IGFBP2, LRG1 and MAPRE1 that was constructed based on the newly diagnosed samples was evaluated in an independent set of pre-diagnostic WHI plasma samples consisting of 32 CRC cases and 32 matched controls drawn within 7 months prior to diagnosis of CRC. This combination rule resulted in an AUC of 0.724, with 41% sensitivity at 95% specificity (Figure 3b), compared with 19% sensitivity at 95% specificity for CEA alone.
Furthermore, CEA, LRG1 and MAPRE1 were each significantly elevated in cases compared to controls (Table 3). IGFBP2 was not significantly elevated in the pre-diagnostic samples, with a mean ratio of 1.27 and p<0.1. Individual markers had AUCs between 0.586 (IGFBP2) and 0.723 (LRG1) (Table 3). Ratios for each marker were lower in the pre-diagnostic samples compared to the newly diagnosed group.

Discussion

The proteomic analysis of nine pools from 180 plasma samples from the WHI cohort collected prior to diagnosis and an equal number of matched controls yielded a set of 6 proteins that were significantly up-regulated in cases compared to controls. Three of these proteins, LRG1, IGFBP2 and ARMET, are known to be secreted whereas MAPRE1, PDIA3 and ENO1 are predominantly intracellular. IGFBP2, LRG1 and MAPRE1 were selected for further characterization and validation based on the availability of ELISA assays. Immunological testing of these three proteins along with CEA in plasmas from newly diagnosed subjects demonstrated significant (p<0.05) elevation of each in cases compared to controls. A linear combination of the four proteins yielded 59% sensitivity at 95% specificity for plasmas from newly diagnosed cases relative to controls indicative of the potential of the marker panel for improved monitoring of CRC. Addition of the three markers to CEA also improved performance in pre-diagnostic samples. Sensitivity was increased from 19% for CEA alone to 41% at 95% specificity for the panel in blood drawn within seven months prior to diagnosis of CRC. Additionally, CEA, LRG1 and MAPRE1 were each significantly elevated in the pre-diagnostic plasmas. IGFBP2 yielded a case-to-control ratio of 1.27 prior to diagnosis...
but was not statistically significant. Pre-diagnostic samples separated by stage showed increased levels of CEA and MAPRE1 in Stage 3/4 cases compared to Stage 1/2 cases (p=0.068 and p=0.120, respectively) (Supplementary Figure 5). For both proteins, only Stage 3/4 cases were significantly higher than controls. LRG1 in Stage 3/4 cases was more significantly elevated compared to controls than Stage 1/2 cases. Our findings suggest that circulating plasma levels of CEA, LRG1 and MAPRE1 may all increase with tumor progression.

Extensive mass spectrometry evaluations of IGFBP2, LRG1 and MAPRE1 in other cancers and inflammatory diseases have been carried out by our group. Protein levels were on average unchanged across multiple experiments in both breast and lung cancer for each of the three proteins. In patients who developed coronary heart disease, MAPRE1 and IGFBP2 were decreased or unchanged in diseased individuals compared to matched controls, while LRG1 was not quantified. CEA was not quantified in any of the MS experiments likely due to its high degree of glycosylation.

A comprehensive proteomic analysis of an Apc Δ580 mouse model was previously performed by our group(29). From that analysis, it was observed that circulating levels of both LRG1 and IGFBP2 were significantly (p<0.05) elevated in tumor bearing mice compared to controls. ENO1 and PDIA3 were also identified in the analysis of mouse plasma samples based on non-cysteine containing peptides, thus lacking quantification, while no peptides from MAPRE1 or ARMET were identified, likely due to their very low abundance in plasma.

Mutation of the APC gene is considered to be one of the initiating events in the development of colorectal adenocarcinoma(30). The mutated form of APC is commonly
truncated, retaining only the N-terminus, resulting in increased protein mobility and altered function(31). MAPRE1 is known to bind to APC and participate in the stabilization of microtubules through interactions with the formin mDia(32). Overexpression of MAPRE1 has been found to induce nuclear accumulation of beta-catenin and activate the beta-catenin/T-cell factor pathway leading to a promotion of cell growth and increase in colony formation(33, 34). Our study demonstrates a significant elevation of circulating MAPRE1 protein in newly diagnosed and pre-diagnostic colorectal cancer plasma samples. Expression of MAPRE1 has been reported to be elevated in tissue from head-and-neck cancer(35), and to correlate with tumor size and associated with poor differentiation in hepatocellular carcinoma(36) tissue. Extensive proteomic analysis of two CRC cell lines, as well as Western blot analysis, resulted in the identification of MAPRE1 in conditioned media. Gene expression data from BioGPS indicated that MAPRE1 was strongly expressed in colorectal adenocarcinoma compared to most other tissues, including normal colon(37). Immunohistochemistry for MAPRE1 in colorectal tumor tissues from Human Protein Atlas(38) demonstrates an increase in cytoplasmic staining compared to normal tissues. Our study has revealed for the first time an association between circulating levels of MAPRE1 and colorectal cancer.

Elevated plasma levels of LRG1 have previously been reported for pancreatic and ovarian cancers(39-41), but not for colorectal cancer. LRG1 is primarily expressed in the liver(37) and has been associated with acute-phase response, being induced by pro-inflammatory cytokines, such as IL-6(23). LRG1 was not observed in proteomic analysis of conditioned media from two colorectal cell lines, suggesting that increased circulating levels are a response to tumor development. Elevated circulating levels have previously
been associated with graft versus host disease(14), as well as autoimmune diseases(42).
LRG1 may be released from neutrophils\textsuperscript{24}(43). LRG1 has also been associated with
TGF\(\beta\) signaling(44), specifically through interaction with TGF\(\beta\) receptor type II(45).
CEA has long been established as a marker for colorectal cancer(46). It is a member of
the immunoglobulin superfamily and has been associated with cancer dissemination(47).
Because of its low sensitivity and specificity, CEA has limited utility in screening or
diagnosis of early stage CRC and has primarily been assayed to determine preoperative
prognosis, and for disease monitoring(6, 48). Plasma levels of CEA are reduced
following surgical removal of cancerous polyps(49-51). Our data suggest that CEA may
have utility for early detection of CRC as part of a panel of markers.
IGFBP2 has been previously investigated as a potential plasma marker for colorectal and
other cancers(52-54) with mostly negative findings(55, 56). In this study, plasma levels
were significantly elevated in newly diagnosed patients, but not in pre-clinical samples,
suggesting that circulating levels of IGFBP2 increase with progressive tumor
development(57). Given the occurrence of IGFBP2 in the conditioned media of CRC
cell lines it is likely that an increase in tumor cell mass contributes to the observed
increase in plasma levels with advanced CRC.
ENO1, PDIA3 and ARMET have all previously been investigated in various cancers
but only ENO1 has been associated with colorectal tumor development(26-28).
Levels of PDIA3 in human plasma were found to be elevated in hepatocellular
carcinoma based on an immunoassay(27) and in gastric cancer based on proteomic
mass spectrometry analysis(28). Our findings suggest that these three proteins may
be elevated in colorectal cancer patient plasma prior to clinical diagnosis of the
disease. These markers may further improve the performance of the four marker panel presented in this study. Multiple steps were employed to facilitate the in-depth, quantitative plasma proteomic profiling in this study: Depletion of the 6 most highly abundant plasma proteins, extensive protein fractionation using reverse phase and anion exchange, and use of heavy and light acrylamide labels for comparison of cases and controls. Using these steps, proteins across 7 orders of magnitude and with concentrations in the pg/mL range have been identified. MS-based discovery of this nature while quantitative, does not recognize post-translational modifications, such as glycosylation that may be cancer-related(58). Most prior discovery studies of blood based biomarkers for early detection have been based on analysis of specimens collected at the time of diagnosis. In contrast, our study relied on plasma samples collected prior to the clinical manifestation of colorectal cancer to identify and validate a panel of markers that could be useful for early detection and identification of subjects at increased risk of developing CRC. The WHI cohort samples used in discovery and validation consist entirely of post-menopausal women, and may not be representative of the general population as a whole. Hormone therapy use, which may alter the circulating levels of some proteins, was not a factor used in matching case and control samples in this study and could have impacted the plasma proteome. However there was no bias in this regard between cases and controls of which we are aware. However, validation data from newly diagnosed patients suggest that levels of the assayed markers are not confounded by gender (Supplementary Figure 6) or hormone therapy. The WHI cohort meets the requirements of Phase 3 of biomarker development as outlined by Pepe,
et al. (10). Because discovery of markers was also done in pre-clinical samples, Phase 1 and 2 were not applicable, which is an advantage of this study. The primary aims of Phase 3, to evaluate the capacity of the biomarkers to detect pre-clinical disease and to define criteria for a positive screening test, were addressed. CEA, MAPRE1 and LRG1 were shown to significantly differentiate pre-clinical cases from matched controls, while IGFBP2 was elevated in pre-clinical cases, but not significantly so. Furthermore, a linear combination of these four markers was established that differentiates pre-clinical cases from controls with 41% sensitivity at 95% specificity.

Mass spectrometry analysis of pre-clinical colorectal cancer compared to matched controls yielded a set of elevated proteins that were further validated by ELISA. Three of these proteins in conjunction with CEA show promise as a pre-clinical test for CRC. Further improvements in sensitivity and specificity based on inclusion of additional markers may ultimately lead to a blood-based test to aid in screening for colorectal cancer.
References


Figure Captions

**Figure 1.** Tryptic peptides identified from MS experiments for IPAS experiments for a) MAPRE1, b) LRG1, c) IGFBP2, d) ENO1, e) ARMET and f) PDIA3. Each line represents peptides identified or quantified in an individual IPAS. The top line in each figure represents theoretical peptides generated by tryptic digestion. Blue peptides indicate non-cysteine containing tryptic peptides, while red peptides are cysteine-containing tryptic peptides that could be quantified from the acrylamide labeling. In the experimental data, blue peptides indicate the peptide was identified, but not quantified, while red peptides indicate the peptide had quantification data from the acrylamide labeling.

**Figure 2.** ELISA results in newly diagnosed samples of a) CEA, b) MAPRE1, c) LRG1, d) IGFBP2. Each circle represents one of 58 individual cases or matched controls. **** indicates $p<0.0001$ based on paired Wilcoxon test.

**Figure 3.** a) ROC analysis of a linear combination of the four markers from a) compared to CEA in newly diagnosed samples. b) ROC analysis of the same linear combination of the four markers in a) compared to CEA in pre-diagnostic samples. Coefficients for combination of the four markers are: CEA: 3.612e-2 ; IGFBP2: 7.052e-3 ; LRG1: 7.263e-5 ; MAPRE1: 2.766e-2.
Table 1. Pre-diagnostic WHI cohort sample characteristics

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<th>Discovery Set</th>
<th>Validation Set</th>
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<tbody>
<tr>
<td></td>
<td>Case</td>
<td>Control</td>
</tr>
<tr>
<td>Number</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Avg. Age</td>
<td>64.9</td>
<td>64.9</td>
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<tr>
<td>Stage I</td>
<td>8 (9%)</td>
<td>-</td>
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<tr>
<td>Stage II</td>
<td>29 (32%)</td>
<td>-</td>
</tr>
<tr>
<td>Stage III</td>
<td>37 (41%)</td>
<td>-</td>
</tr>
<tr>
<td>Stage IV</td>
<td>16 (18%)</td>
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<tr>
<td>Caucasian</td>
<td>75 (83%)</td>
<td>75 (83%)</td>
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<tr>
<td>African American</td>
<td>10 (11%)</td>
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<tr>
<td>Asian</td>
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<td>3 (3%)</td>
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<td>Hispanic</td>
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<td>1 (1%)</td>
</tr>
<tr>
<td>Other</td>
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<td>1 (3%)</td>
</tr>
<tr>
<td>Missing</td>
<td>1 (1%)</td>
<td></td>
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<tr>
<td>Avg. Days to Diagnosis</td>
<td>245</td>
<td>-</td>
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Table 2. Proteins with significantly elevated levels in plasmas collected prior to diagnosis of CRC compared to matched controls from the WHI cohort that did not develop CRC during the period of follow-up.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Average Case/Control Ratio</th>
<th>p-Value</th>
<th>FDR</th>
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<tr>
<td>MAPRE1</td>
<td>Microtubule-associated protein rp/eb family member 1</td>
<td>4.52</td>
<td>0.019</td>
<td>0.763</td>
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<tr>
<td>PDIA3</td>
<td>Protein disulfide-isomerase a3</td>
<td>1.51</td>
<td>0.027</td>
<td>0.471</td>
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<td>IGFBP2</td>
<td>Insulin-like growth factor-binding protein 2</td>
<td>1.20</td>
<td>0.027</td>
<td>0.420</td>
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<td>ENO1</td>
<td>Alpha-enolase</td>
<td>1.96</td>
<td>0.029</td>
<td>0.713</td>
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<td>ARMET</td>
<td>Mesencephalic astrocyte-derived neurotrophic factor</td>
<td>1.64</td>
<td>0.043</td>
<td>0.417</td>
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<td>LRG1</td>
<td>Leucine-rich alpha-2-glycoprotein</td>
<td>1.35</td>
<td>0.046</td>
<td>0.487</td>
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</table>
**Table 3.** ELISA summary for individual proteins in newly diagnosed and pre-diagnostic colorectal cancer samples compared to matched healthy controls. Newly diagnosed samples are both male and female. Pre-diagnostic WHI samples are all female.

<table>
<thead>
<tr>
<th></th>
<th>Newly Diagnosed Samples</th>
<th>Pre-Diagnostic Samples</th>
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<tr>
<td></td>
<td>Ratio</td>
<td>p-Value</td>
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<tr>
<td>CEA</td>
<td>45.76</td>
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<tr>
<td>IGFBP2</td>
<td>1.66</td>
<td>6.2E-05</td>
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<tr>
<td>LRG1</td>
<td>1.60</td>
<td>5.0E-05</td>
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<tr>
<td>MAPRE1</td>
<td>10.79</td>
<td>3.9E-07</td>
</tr>
</tbody>
</table>
Figure 1c
Figure 1e
Figure 2a
Figure 2b
Figure 2c
Figure 2d
Figure 3a
Figure 3b
Cancer Prevention Research

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