Evaluation of a 5-Marker Blood Test for Colorectal Cancer Early Detection in a Colorectal Cancer Screening Setting

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Abstract

Purpose: In initial studies that included colorectal cancer patients undergoing diagnostic colonoscopy, we had identified a serum marker combination able to detect colorectal cancer with similar diagnostic performance as fecal immunochemical test (FIT). In this study, we aimed to validate the results in participants with similar diagnostic performance as fecal immunochemical test (FIT). In this study, we aimed to validate the results in participants similar to the target population of screening. For osteopontin, seprase, and ferritin, the diagnostic performance in the screening setting was reduced compared with previous studies in diagnostic settings while CEA and anti-p53 showed similar diagnostic performance in both settings.

Conclusions: Performance of the 5-marker blood test under screening conditions is inferior to FIT even though it is still comparable with the performance of gFOBT. CEA and anti-p53 could contribute to the development of a multiple marker blood-based test for early detection of colorectal cancer. Clin Cancer Res; 22(7): 1725–33. ©2015 AACR.

Introduction

With over 1.3 million new cancer cases worldwide and almost 700,000 deaths each year, colorectal cancer is one of the most common cancers (1). Because of the slow progression from detectable and curable precancerous lesions to colorectal cancer and the strong dependence of prognosis on stage at diagnosis, early detection of colorectal cancer has great potential to reduce the burden of this disease (2–4). Colonoscopy is the gold standard for detecting colorectal cancer and its precursors, but its routine use is impaired by high costs, limited capacities, and typically lower adherence (5, 6). Noninvasive stool tests are an attractive alternative for colorectal cancer screening due to their low cost and suitability for home use or in a primary care setting. However, the aversion to stool handling for gFOBT and FITs may limit the acceptance of stool tests (7). Blood-based tests may be more readily accepted by patients for many medical conditions, and surveys conducted in individuals eligible for colorectal cancer screening showed a strong preference for collection of blood over feces (8). While the development of a simple and convenient blood test with diagnostic performance comparable with FIT could contribute to improvement in the acceptance of colorectal cancer screening, the diagnostic performance of currently existing blood tests is insufficient for their routine use (9).

The identification of a blood-based multimarker panel for detection of colorectal cancer in a marker identification program (MIP) study has been previously described (10). While most controls were recruited in a screening setting, colorectal cancer cases recruited from surgery centers were used to enrich the number of cancer cases due to the low prevalence of colorectal cancer in the screening population (10, 11). A multivariate analysis of 60 candidate biomarkers identified a 6-marker panel consisting of CEA, ferritin, seprase, osteopontin, anti-p53 autoantibody, and CYFRA-21-1. With sensitivity and specificity of 69.6% and 95.0%, respectively, for the detection of colorectal cancer, the diagnostic performance of the panel was comparable with the performance of FIT (10).

To validate this result in the target population of screening, we combined 5 of the 6 candidate biomarkers to a 5-marker blood test.
Translational Relevance

Blood tests for early detection of colorectal cancer would be a highly attractive alternative to endoscopic examinations and stool tests. In the last decade, many candidate biomarkers were identified in studies that included symptomatic colorectal cancer cases recruited in clinics. However, the majority of these biomarkers failed in subsequent studies under screening conditions which stresses the importance of the study setting for biomarker discovery and validation. Here, we tested 1,660 blood samples from participants of screening colonoscopy with a 5-marker blood test [carcinoembryonic antigen (CEA) + anti-p53 + osteopontin + seprase + ferritin]. The diagnostic performance of the 5-marker test was comparable with guaiac-based fecal occult blood test (gFOBT) but inferior to fecal immunochemical test (FIT). Of note, a combination of anti-p53 and CEA was sufficient to reach the same diagnostic performance under screening conditions as the whole 5-marker panel suggesting preference for these two markers for future multimarker panel development.

Materials and Methods

Study design and sample selection

Samples were drawn from the BliTz study collective. BliTz (Begleitende Evaluierung innovativer Testverfahren zur Darmkrebs-Früherkennung) is an ongoing prospective screening study conducted in cooperation with more than 20 gastrointestinal practices in southwestern Germany. Detailed information on the BliTz study can be found elsewhere (12–14). Briefly, since the end of 2005 precolonoscopy stool and blood samples from more than 7,000 participants of screening colonoscopy were collected. After colonoscopy, basic demographic and clinical data were extracted from colonoscopy and histology reports in a standardized manner by trained research assistants who were blinded to blood and stool test results. Cancer stages were classified according to the UICC (Union for International Cancer Control) classification and advanced adenomas were defined as adenomas with at least one of the following features: 1 cm in size, tubulovillous or villous components, and high-grade dysplasia. Further patient data were collected with a short patient questionnaire. Personal and clinical data were stored separately in access databases for reasons of privacy protection. The study was approved by the Ethics Committee of the University of Heidelberg (Heidelberg, Germany) and informed consent was obtained from all participants. Auditing was conducted by the coordinating study center at the German Cancer Research Center and by the Coordination Centre for Clinical Trials (KKS) Heidelberg (Heidelberg, Germany). For the current analysis, participants with colorectal cancer (N = 36), participants with carcinoma in situ (CIS, N = 4), and participants with advanced adenoma (N = 420) were compared with controls without colorectal neoplasms (N = 1,200). While the colorectal cancer and the CIS group were comprised of all available colorectal cancer/CIS patients from the BliTz study recruited until the February 20, 2013, for the advanced adenoma and the control group, representative samples were selected from the available BliTz participants. BliTz participants whose blood was taken after colonoscopy or for whom the date of blood withdrawal was unknown were excluded. Furthermore, study participants with a history of colorectal cancer or inflammatory bowel disease, participants with a previous colonoscopy within the last 5 years, and persons ages below 50 or over 79 years were excluded, because these participants would typically not be considered to be the target population for colorectal cancer screening. In the control group also participants with insufficient bowel preparation before colonoscopy or incomplete colonoscopy were excluded to avoid false-negative colonoscopy results. Because this study was conducted in a true screening population in which colorectal cancer and adenoma patients are expected to be, on average, slightly older and to include a larger proportion of men, we did not match for these factors as this might lead to biased estimates of specificity in such a setting (15).

Handling of blood samples

After blood withdrawal in the gastrointestinal practices, serum samples were incubated at room temperature for 30 to 60 minutes to allow blood clotting and centrifuged at 2,000 to 2,500 × g for 10 minutes. Then they were transported to the DKFZ laboratory in a cold chain (medium transport time: 1 day), centrifuged again, aliquoted, and stored at −80°C. For testing, the serum samples were randomized and shipped on dry ice to Roche Diagnostics GmbH. There was not more than one freeze–thaw cycle for each sample. The laboratory staff was blind to any information regarding the study population.

Immunoassays

The five biomarkers CEA, ferritin, seprase, osteopontin, and anti-p53 antibody were measured quantitatively on a cobas e601 platform. The assays for each marker are designed as sandwich assays based on the streptavidin–biotin technology. The capture antibodies are biotinylated and bind to streptavidin-coated microparticles. The secondary antibodies, covalently linked to Ruthenium complexes, are used for electrochemiluminescent detection (16).

For CEA and ferritin, the commercial tests Elecsys CEA (Roche Diagnostics GmbH, catalog number: 11731629.322) and Elecsys Ferritin (Roche Diagnostics GmbH, catalog number: 04491785.190) were used according to the manufacturer’s instructions. Calibration was performed with the CEA CalSet (Roche Diagnostics GmbH, catalog number: 11731645322) and the Ferritin CalSet (Roche Diagnostics GmbH, catalog number: 03737586.190) in accordance with the package inserts. Reagents for the quantitative analysis of seprase, osteopontin, and anti-p53 antibody for the cobas e platform were available as prototypes at Roche Diagnostics. Performance characteristics for prototype assays are expected to be similar to those seen for...
commercial Elecsys assays, that is, repeatability CV ≤ 5%–6%, intermediate precision/total imprecision CV ≤ 7%–8%, dilution linearity within ±10%. For the prototype calibrators M-Cal-Seprase, M-Cal-Osteopontin, and M-Cal-anti-p53, a full calibration was performed.

**gFOBTs and FITs**

In the context of the BliTz study, different stool tests for the early detection of colorectal cancer were evaluated. For all except two participants included into this study, FIT results were available and for most study participants (HemOccult, Beckman Coulter GmbH) gFOBT results were also available. While participants recruited before February 2009 were tested by the quantitative FIT RIDASCREEN Hemoglobin (R-Biopharm AG) as described elsewhere (17), for participants recruited since February 2009 the quantitative FIT FOB Gold (Sentinel Diagnostics) was used. Until January 2012, BliTz participants collected native stool samples that were immediately frozen and thawed once before conducting the FIT according to the manufacturers’ instructions in a central laboratory. Since the end of January 2012, participants directly used the buffer-filled stool collection tubes from Sentinel Diagnostics for sample collection and mailed them to the central laboratory for analysis according to the manufacturer’s instructions. For all three FIT conditions (frozen stool + RIDASCREEN, frozen stool + FOB Gold, fresh stool in buffer-filled sentinel tubes + FOB Gold), we calculated cutoffs for test positivity based on all available BlitZ controls with this FIT condition. At 96% specificity, cutoffs were 9.6 μg hemoglobin/g stool for the RIDASCREEN test, 12.5 μg hemoglobin/g stool for the FOB Gold test with frozen stool samples, and 15.3 μg hemoglobin/g stool for the FOB Gold test with fresh stool samples collected in buffer-filled Sentinel tubes.

**Data processing and statistical analysis**

For processing of the data obtained from the immunoassays, the evaluation software OASE was used. For further data analyses, statistical software [R version 3.1.0 (18) and SAS version 9.2 (SAS Institute)] was used.

Basic demographic characteristics in the study population (sex, age, and UICC stage) were summarized. The results of the five individual tests were combined into one single diagnostic result (the “score”) at the biostatistics department of Roche Diagnostics using a defined algorithm with a predefined cutoff. This algorithm was selected by penalized LASSO regression on data of the MIP study (10) and reoptimized in a second large panel with screening controls and enriched cases (CT study). For the reoptimized algorithm, the MIP study marker CYFRA 21 was dispensable. That is why this marker was not tested in the BliTz study anymore. An algorithm for the marker combination CEA + anti-p53 was derived from a logistic regression model trained on data from the CT study. Sociodemographic characteristics of this study collective can be found in Supplementary Table S1.

Univariate marker results and results for marker combinations were compared between participants with colorectal cancer and controls and between participants with advanced adenomas and controls. Clinical performance [sensitivity and specificity with exact 95% confidence intervals (CI)] and ROC curves for detection of colorectal cancer and advanced adenoma were determined. In addition to analyses in the whole study population, we performed stage-specific analyses. AUCs were compared by the DeLong method with the R package "pROC" (19). For patients with gFOBT and FIT results, we also evaluated agreement between the 5-marker blood test and the stool tests.

**Results**

**Characterization of the study population**

For validation of the 5-marker blood test, 1,660 participants (36 patients with invasive colorectal cancer, 4 CIS patients, 420 advanced adenoma patients, and 1,200 participants free of neoplasms) were selected from eligible participants of the colorectal cancer screening study BliTz as described in the STAndards for the Reporting of Diagnostic accuracy studies (STARD) diagram (see Fig. 1). Sociodemographic characteristics of all participants with valid measurement results (n = 1,656) are summarized in Table 1. As expected, for a true colorectal cancer screening setting, the average age among colorectal cancer, CIS, and advanced adenoma cases is slightly higher than among controls (mean ± SD: 66.0 ± 6.2, 63.0 ± 5.3, and 63.6 ± 6.7 vs. 62.0 ± 6.1 years). Also, the proportion of men is higher among cases than among controls (colorectal cancer, 72.2% men; CIS, 75.0% men; advanced adenomas, 64.7% men; controls, 45.5% men). Among the group of colorectal cancer patients, early-stage cancers (UICC stage I/II) were equally common as late-stage cancers (UICC stage III/IV).

**Diagnostic performance of the original marker panel**

For blood samples from all except four subjects (one advanced adenoma patient and three controls) valid measurements for all five Elecsys Assays (CEA, ferritin, seprase, osteopontin, and anti-p53) could be obtained. We used a predefined algorithm obtained in the MIP study and validated in the CT study to combine the results from the five Elecsys Assays into a single prediction score. ROC curve analysis revealed an AUC of 0.78 (95% CI, 0.68–0.87) for the discrimination of colorectal cancer patients (stage 1–IV) and controls and an AUC of 0.56 (95% CI, 0.53–0.59) for the discrimination of advanced adenoma patients and controls (see Fig. 2). In a sensitivity analysis including 4 CIS patients, the diagnostic performance for the detection of colorectal cancer (stage 0–IV) was slightly worse with an AUC of 0.76 (95% CI, 0.67–0.85).

When using a cutoff at 90% specificity in the CT study, the 5-marker combination yielded sensitivities of 44 (95% CI, 28–62) and 12 (95% CI, 9–15) for colorectal cancer and advanced adenomas at a specificity of 94 (95% CI, 92–95). When adjusting the cutoffs to yield specificities of 80%, 90%, and 95% in the BliTz collective, sensitivities for the detection of colorectal cancer were 64%, 50%, and 42%. These values are lower than the sensitivities observed in the MIP study (10) and the CT study (see Supplementary Table S2). Sensitivities for advanced adenomas were below 30% even at cutoffs yielding 80% specificity (see Table 2).

With AUCs of 0.80 (95% CI, 0.67–0.93) and 0.75 (95% CI, 0.62–0.88), the ability of the 5-marker blood test to detect early- and late-stage cancer was similar (P = 0.57) (see Table 2).

**Diagnostic performance of single markers**

To further evaluate the loss of performance of the 5-marker panel in the BliTz study collective, we compared the univariate results for CEA, ferritin, seprase, osteopontin, and anti-p53 in the BliTz and in the CT study. Interestingly, two of the five markers...
(CEA and anti-p53) showed very similar diagnostic performance in both studies [AUC in BliTz, 0.84 (95% CI, 0.78–0.90) and 0.57 (95% CI, 0.51–0.63); AUC in CT study, 0.77 (95% CI, 0.73–0.81) and 0.59 (95% CI, 0.56–0.62)] while the other markers did not (see Fig. 3A–E). The largest decrease of diagnostic performance was seen for seprase with an AUC of 0.78 (95% CI, 0.74–0.82) in the CT study and an AUC of 0.60 (95% CI, 0.49–0.70) in the BliTz study.

Diagnostic performance of a 2-marker combination
Because of the poor univariate results for seprase, osteopontin, and ferritin in the BliTz study collective, we decided to perform an exploratory evaluation of the marker combination CEA + anti-p53. For training of the algorithm, data from the CT study were used. In the BliTz study, the 2-marker combination reached an AUC of 0.85 (95% CI, 0.78–0.91) for the detection of colorectal cancer (see Fig. 3F) and an AUC of 0.56 (95% CI, 0.53–0.59) for the
detection of advanced adenomas. When adjusting the specificity to 80%, 90%, and 95%, the sensitivities for colorectal cancer detection were 67%, 58%, and 47% for the 2-marker combination. For the same specificities, the sensitivities of CEA alone were 62%, 50%, and 40%.

Comparison of the 5-marker blood test with stool tests

For 23 colorectal cancer cases, 301 advanced adenoma cases, and 899 controls, results of the 5-marker blood test and both stool tests (gFOBT and FIT) were available. Table 3 shows the diagnostic performance of all tests in this subpopulation. For reasons of comparison, the specificities of the FIT and the 5-marker blood test were adjusted to the specificity of the gFOBT (96%). At this specificity, both the 5-marker blood test and the gFOBT could identify 39% of all colorectal cancer cases. Of note, the gFOBT and the 5-marker blood test primarily detected different colorectal cancer patients which led to an increased sensitivity of 65% (at minimal loss of specificity) when combining gFOBT and blood test results. For advanced adenomas, the sensitivities of the 5-marker blood test, the gFOBT, and the combination of both tests were 8%, 4%, and 12%. With sensitivities of 78% for colorectal cancer and 28% for advanced adenomas, the FIT was superior to the other tests and its combination with the 5-marker panel could only slightly further increase sensitivities (83% for colorectal cancer, 35% for advanced adenomas) at the cost of a lower specificity (92%).

Discussion

In this study, we evaluated a 5-marker blood test for colorectal cancer early detection in a large screening study. The AUCs for the detection of colorectal cancer and advanced adenomas were 0.78 (95% CI, 0.68–0.87) and 0.56 (95% CI, 0.53–0.59), respectively. At specificities of 80%, 90%, and 95%, the sensitivities for the detection of colorectal cancer reached 64%, 50%, and 42%, respectively. Early-stage cancers were detected at least as well as late-stage cancers. Of note, two of the five markers (CEA and anti-p53) were sufficient to achieve a similar or even better diagnostic performance.
performance for colorectal cancer (AUC, 0.85; 95% CI, 0.78–0.91). In a subsample of participants with gFOBT, FIT, and blood test results, we directly compared the performance of these tests. With a sensitivity of 39% at a specificity of 96%, gFOBT and the 5-marker blood test performed equally well and sensitivity increased to 65% when both tests were combined. Nevertheless, both tests and their combination were outperformed by FIT.

In our previous MIP study, a 6-marker blood test (5-marker panel with the addition of CYFRA21–1) was able to detect colorectal cancer patients with a sensitivity of 69.6% at a specificity of 95%, which was similar to the diagnostic performance of FIT in the MIP study (10). In the CT study that was used to refine the algorithm for the 5-marker blood test, the observed sensitivity of the 5-marker blood test was 68.1% at 95% specificity. The obvious drop of diagnostic performance of the 5-marker blood test in participants selected from the BliTz study collective may be explained by differences in the study populations. While BliTz is a real screening study in which all cases were recruited before diagnosis at screening colonoscopy, colorectal cancer cases in the MIP and CT study had to be enriched with patients recruited at surgery units. For clinically recruited colorectal cancer cases, there is the possibility that blood marker levels are altered by diagnostic or therapeutic interventions or lifestyle changes in response to the colorectal cancer diagnosis. Furthermore, in contrast to screening settings, in clinical settings it cannot be guaranteed that blood withdrawal and blood storage conditions at recruitment site are exactly the same for cases and controls. Last but not the least, colorectal cancer patients recruited in clinical settings are often in a more advanced stage and more often present symptoms than colorectal cancer patients diagnosed at screening colonoscopy (20).

Our univariate analyses suggest that not all markers of the 5-marker blood test are as prone to study setting issues as others. With an AUC of 0.84, the diagnostic performance of CEA was even better than in the CT study (AUC, 0.77) and for anti-p53, the AUCs in both studies were quite similar (AUC in BliTz, 0.57; AUC in CT study, 0.59). These results suggest that the observed diagnostic performance for CEA and anti-p53 indeed represents true cancer-specific differences between cases and controls. For ferritin, osteopontin, and sepsis, other factors might have contributed to the previously observed good diagnostic performance for colorectal cancer detection. Serum ferritin levels, for instance, can be influenced by age, sex, body mass index, acute or chronic inflammation, and aspirin use in addition to cancer (21). For colorectal cancer, the situation is especially complex, because possible positive correlations between cancer-specific processes and ferritin, as found for other cancers (21), might be antagonized by iron deficiency anemia caused by chronic gastrointestinal bleedings.

The sensitivity of anti-p53 is limited due to the fact that not all colorectal cancer patients have p53 mutations and not all patients with p53 mutations produce antibodies against this tumor suppressor protein (22). Nevertheless, the remarkably high specificity of anti-p53 for cancer, which can also be seen in the very steep slope of the left part of its ROC curve (see Fig. 3B), makes it possible to increase the sensitivity of conventional tumor markers without reducing specificity to a relevant extent when anti-p53 is added in a marker combination (23). The combined use of CEA and anti-p53 for colorectal cancer detection has been evaluated in earlier studies, and sensitivities between 33% and 73% have been reported (22–26). However, none of these studies was performed in a screening setting and only Kojima and colleagues reported specificities for this marker combination. In their newer and larger study from 2011, a sensitivity of 48% at 93% specificity for the detection of colorectal cancer was reached (25), which is very similar to our findings in participants of screening colonoscopy. It should be stated that both CEA and anti-p53 are not cancer type–specific and have been found in patients with other cancers like lung cancer (27, 28). Thus, there is a possibility that some of the controls with false-positive test results actually are persons with an undiscovered other malignancy.

With 47% and 6% sensitivity at 95% specificity, the capability of the marker combination CEA + anti-p53 to detect colorectal cancer and advanced adenomas was comparable or even superior to Epi proColon, which, to our knowledge, is the only prospectively evaluated blood test for early detection of colorectal cancer so far (29). Although the 2-marker combination CEA + anti-p53 alone cannot compete with the FIT, it appears plausible that a combination with further blood biomarkers, such as DNA methylation markers (30), miRNA markers (31), autoantibody markers (24), or protein markers (32, 33) might increase the diagnostic performance sufficiently for an application in mass screening.

Blood tests seem to be better accepted in public than stool tests. For instance, in a study by Adler and colleagues, over 100 persons that refused to participate in screening colonoscopy were offered a choice of a blood-based or a stool-based colorectal cancer early detection test and while 83% of the participants picked the blood test, only 15% picked the stool test (34). One major advantage of FIT over current blood tests, in addition to the better diagnostic performance for detection of colorectal cancer, is its ability to detect a relevant proportion of advanced adenomas. Stool tests might have a larger potential to capture localized tumor effects in general such as excretion of blood or components of tumor cells with stool which would be hard, if not impossible, to detect by blood tests, in particular, at early tumor stage and for precursors of colorectal cancer. In our
analyses, the diagnostic performance for the detection of advanced adenomas was poor and the diagnostic performance for the detection of non-advanced adenomas is expected to be even worse. Non-advanced adenomas were deliberately not included in our analyses as their transition rates to more advanced neoplasms are low (35) and there is an ongoing debate whether they should be considered as one of the target lesions for colorectal cancer screening or not (36). For the development of future blood tests for early detection of colorectal cancer, it would be beneficial to identify markers that also

Figure 3.
A–E, Univariate analysis: Performance of CEA, anti-p53, osteopontin, ferritin and seprase in the BITZ and in the CT study. F, Diagnostic performance of the 2-marker combination anti-p53 + CEA in the BITZ study.
The combination 5-marker blood test in colorectal cancer patients and controls under screening conditions suggests the potential of CEA and anti-p53 to discriminate colorectal cancer early detection in participants selected from a population-based study. However, the performance estimates for the 5-marker blood test dropped from those for individual markers for prognosis or monitoring colorectal cancer patients. In conclusion, the validation of a 5-marker blood test for colorectal cancer early detection in participants selected from a screening study collective uncovered decreased diagnostic performance for the markers ferritin, osteopontin, and seprase, compared with previous evaluations in studies conducted among cancer patients and controls under screening conditions suggesting their potential to contribute to the development of a multi-marker blood-based test for early detection of colorectal cancer.

Disclosure of Potential Conflicts of Interest
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