Simultaneous Multianalyte Immunoassay Measurement of Five Serum Tumor Markers in the Detection of Colorectal Cancer*

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Abstract. *Several serum tumor markers (STMs) have been proposed for the diagnosis of colorectal cancer (CRC), but their detection should be combined to increase accuracy. The measurement of a serum biomarker panel may improve the diagnostic value of single STM and a multianalyte immunoassay approach can shorten assay time and lower sample consumption. The aim of this study was to determine whether the simultaneous multianalyte immunoassay is useful for early detection of CRC. We measured a panel of five STMs namely, carcinoembryonic antigen (CEA), cancer antigen (CA) 19-9 and 72-4, cytokeratin fragment (CYFRA) 21-1, and osteopontin, in a selected homogeneous population of 102 consecutive patients (median age 66 years, range 42-75 years) with Dukes B, G1-2, colorectal adenocarcinoma (cases) and in a group of 99 age- and sex-matched patients suffering from confirmed benign colorectal diseases (controls). Overall, 141 (70.1%) men and 60 (29.9%) women were studied. The highest sensitivity was 45.1% (osteopontin), while the highest specificity was 90.9% (CEA). The accuracy was lower, ranging from 24.9% (CA 19-9) to 67.2% (CEA). CYFRA 21-1 and CA 72-4 had similar sensitivity (35.3% and 31.4%, respectively), but a significantly different specificity (37.4% vs. 89.9%). A combination of the five markers achieved 74.1% sensitivity and 94.3% specificity. In conclusion, in patients with CRC all single STMs show low sensitivity and specificity, while the simultaneous measurement of a panel of STMs may increase the diagnostic accuracy. When the sample volume is limited, the multianalyte immunoassay can be a reliable tool for studying patients undergoing laboratory screening for CRC.*

Colorectal cancer (CRC) still remains the second and third leading cause of cancer death in the USA and UK, respectively (1). However, both the mortality and incidence rate for CRC have decreased over the last two decades, likely because of advances in early detection and treatments (2, 3).

Several serum tumor markers (STMs) have been proposed for the diagnosis of CRC, but their detection should be combined to increase accuracy. It has been suggested that serum biomarker-based CRC screening should be combined with fecal protein markers, including immunologic fecal occult blood test and the tissue inhibitor of metalloproteinase-1 (TIMP-1) and S100A12, to achieve better results (4). However, measurement of a serum biomarker panel may improve the diagnostic value of single STM and a multianalyte immunoassay approach is useful to shorten assay time, lower sample consumption and reduce overall cost (5, 6).

The aim of this study was to determine whether a simultaneous multianalyte immunoassay technology can be useful for early detection of CRC.

Patients and Methods

We measured a panel of five STMs namely, carcinoembryonic antigen (CEA), cancer antigen (CA) 19-9 and 72-4, cytokeratin fragment (CYFRA) 21-1, and osteopontin in a selected homogeneous population of 102 consecutive patients (median age 66 years, range 42-75 years) with confirmed Dukes B, G1-2, colorectal adenocarcinoma (cases), and in a group of 99 age- and sex-matched patients suffering from confirmed benign colorectal diseases (controls). Overall, 141 (70.1%) men and 60 (29.9%) women were studied.

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Table I. *Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the single serum tumor markers, odds ratio (OR) estimates, associated 95% confidence interval (CI) and relative p-value obtained using the Pearson's χ^2 test.*

Marker	TP	FN	TN	FP	Sensitivity	Specificity	PPV	NPV	Accuracy	OR	95% CI	*p*-value
CEA	45	57	90	9	44.1%	90.9%	83.3%	61.2%	67.2%	7.89	3.59-17.37	<0.0001
CYFRA 21-1	36	66	37	62	35.3%	37.4%	36.7%	35.9%	36.3%	0.33	1.18-0.58	<0.0001
Osteopontin	46	56	31	68	45.1%	31.3%	40.4%	35.6%	38.3%	0.37	0.21-0.67	0.007
CA 72-4	32	70	89	10	31.4%	89.9%	76.2%	56.0%	60.1%	4.07	1.87-8.84	0.002
CA 19-9	23	79	27	72	22.5%	27.3%	24.2%	25.5%	24.9%	0.11	0.06-0.21	<0.0001

CEA, Carcinoembryonic antigen; CYFRA 21-1, cytokeratin fragment 21-1; CA 72-4, cancer antigen 72-4; CA 19-9, cancer antigen 19-9; TP, true-positive; FN, false-negative; TN, true-negative; FP, false-positive.

According to the American Joint Committee on Cancer, Dukes B tumors invade the *muscolaris propria* into the subserosa (T3) or other organs or structures (T4), with no regional lymph node metastasis (N0) or distant metastasis (M0), while histologic grade (G) 1 was considered well-differentiated and G2 moderately differentiated (7). Written informed consent was obtained from all the participants. Blood samples were obtained from all participants following overnight fasting, were assayed in duplicates and the average was compared with the manufacturers' standard curves.

A multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) array technology was used and the five microELISA assays were developed using commercially available antibodies. The antigens CEA, CA 19-9, CA 72-4, CYFRA and human osteopontin were analyzed simultaneously (8). The same CEA and osteopontin antibodies used in the microELISA format were also tested in a conventional ELISA format, based on two-sites monoclonal antibody against CEA and rabbit polyclonal antibodies against peptides 288-304 and 211-228 of human osteopontin, respectively (9, 10). ADVIA Centaur® Immunoassay system (Bayer Healthcare, Tarrytown, NY, USA) was used to determine if there was any difference in results between the microELISA.

The obtained cut-off limit values (at 95% specificity) were the following: 4.9 ng/ml CEA; 32.6 U/ml CA 19-9; 8.5 U/ml CA72-4, 2.7 ng/ml CYFRA 21-1; and 811.9 pmol/ml osteopontin.

Sensitivity was defined as true-positives (TP)/TP + false-negatives (FN); specificity as true-negatives (TN)/TN + false-positives (FP); positive predictive value (PPV) as TP/(TP+FP); negative predictive value (NPV) as TN/(TN+FN), and accuracy as (TN+TP)/overall patients. Odds ratio (OR) estimates and the associated 95% confidence interval (CI) were obtained. Testing ranges were determined by regression statistics obtained from a comparison of study methods used to calculate the reference limits of a new method (11). The coefficient of variation (R) of test samples at different dilutions calculation was used to determine the interassay precision. The Pearson's chi-square (χ^2) test and the relative *p*-value were also calculated.

Results

Regression analysis indicated that the two micro-ELISA assays for CEA and osteopontin were not statistically different from the conventional assays (R=0.96 and 0.98, respectively; $p<0.0001$). The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of single STMs are reported in Table I.

None of the tested STMs was sufficiently sensitive for use as a screening marker at 95% specificity. The highest sensitivity was 45.1% for osteopontin and 44.1% (CEA), while the highest specificity was 90.9% for CEA. The accuracy was lower, ranging from 24.9% (CA 19-9) to 67.2% (CEA).Thus, in the diagnosis of CRC, CEA and CA 72-4 were the most useful single STMs.

CYFRA 21-1 and CA 72-4 had similar sensitivity (35.3% and 31.4%, respectively), but a significantly different specificity (37.4% *vs*. 89.9%). A combination of the five markers achieved 74.1% sensitivity and 94.3% specificity.

Discussion

STMs are chemical substances generated by the reactions of the human body to certain tumors, or are expressed and synthesized by genes in tumor cells and include proteins (glycoproteins), enzymes (isoenzymes), or peptide hormones, which may reveal the presence of cancer (12, 13). Increased serum levels of STMs are significantly associated with certain tumor types (14). Thus, the elevation of STM levels in human serum can be useful for early diagnosis of cancer or recurrence and for monitoring the curative effect of chemotherapy (15).

CEA is one of the most widely used tumor markers, especially for patients with CRC, and the most useful application of CEA is in the detection of liver metastasis. Several studies suggested the usefulness of a combination of CEA plus CA 72-4 and CA 19-9 (16, 17).

CYFRA 21-1 is a marker of advanced urothelial carcinoma of the bladder, as well of stage I-II non-small cell lung carcinoma (18, 19). However, in combination with other STMs, it has also been tested in the detection of CRC and the performance of the marker combination was comparable with fecal immunochemical testing (20, 21).

Osteopontin is a phosphoprotein associated with tumor progression in several types of solid tumors, including CRC (22). Its expression is strongly elevated in patients with metastatic disease and inversely correlates with the interval between diagnosis and resection of colorectal liver metastases

(23). Moreover, osteopontin contributes to CRC development and progression, regulating several tumor functions (24).

Various approaches have been proposed to perform multianalyte immunoassays, including label-free immunoassays and labeled probe methods, which are difficult to develop because of the lack of a detectable protein signal or a signal too weak to quantify the trace amount of analytes (25). Multilabel and spatially resolved assays can offer amplified detection signals for multianalyte immunoassays (26). Unfortunately, they need several labels, such as radioisotopes, fluorescent dyes, enzymes, metal ions, or quantum dots, which limits their application (24, 27, 28). The electrochemical immunoassay has the advantages of small analyte volume required, low detection limit, simple instrumentation, and minimal manipulation, due to a miniaturized assay system (29).

Conclusion

In patients with CRC all single STMs show low sensitivity and specificity, while the simultaneous measurement of a panel of STMs may increase the diagnostic accuracy (14). When the sample volume is limited, the multianalyte immunoassay can be a reliable tool for studying patients undergoing laboratory screening for CRC.

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