Multiple Markers of Malignancy in Sera of Patients with Colorectal Carcinoma: Preliminary Clinical Studies

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Eleven potential biochemical markers were measured in serum from 33 patients with malignant and 13 with benign colorectal disease: four isoenzymes (creatine kinase-BB, homoarginine-sensitive alkaline phosphatase, salivary-type amylase, and macro-creatinine kinase type 2), five specific proteins (ferritin, α1-acid glycoprotein, C-reactive protein, α1-antitrypsin, and ceruloplasmin), one oncofetal antigen (carcinoembryonic antigen, CEA), and one hormone (beta human chorionic gonadotropin). The sensitivity of individual markers for the detection of early-stage malignancy (n = 11) ranged from 0% to 64% (CEA 18%); for late-stage colon malignancy (n = 12) from 8% to 83% (CEA 83%). Specificity in patients (n = 10) with benign intestinal disease ranged from 80% to 100% (CEA 100%). The five most-sensitive markers—C-reactive protein, α1-glycoprotein, CEA, macrocreatinine kinase type 2, and homoarginine-sensitive alkaline phosphatase—were selected for use as a "colon panel." In retrospective comparison, use of the colon panel instead of CEA alone increased sensitivity by 17% and 64% for late and early-stage cancer, respectively; specificity, however, decreased by 30%, but should improve with serial testing.

Additional Keyphrases: cancer • sensitivity and specificity • creatine kinase-BB • homoarginine-sensitive alkaline phosphatase • salivary-type amylase • macro-creatinine kinase (type 2) • ferritin • α1-acid glycoprotein • C-reactive protein • α1-antitrypsin • ceruloplasmin • carcinoembryonic antigen • chorionic gonadotropin • colon panel

The search for a unique biochemical "marker" of malignancy has been pursued for decades by biochemists and immunologists. Although a wide variety of biochemical substances (oncofetal proteins, isoenzymes, and hormones) have been assayed in connection with the management of patients with late-stage neoplastic disease, no single marker has proven to be a sensitive and specific indicator of early-stage malignancy.

A promising approach to overcoming the nonspecificity and insensitivity of single tumor markers is the simultaneous assay of several markers (1), based on the premise that cancer cells are biochemically heterogeneous and may synthesize a broad spectrum of possible tumor markers. To avoid the possibility of missing a potential cell marker, a battery of assays might offer the best opportunity to find one or more markers of malignant disease.

Previous reports describing the use of multiple markers have been limited, but the clinical utility of this multiple approach has recently been investigated in breast (2, 3), colon (4), ovarian (5), and testicular (6) tumors. These studies suggest that combinations of certain markers can assist in the detection and monitoring of certain types of malignancy.

We evaluated 11 potential serum markers in malignancy; α1-acid glycoprotein (AAG), C-reactive protein (CRP), α1-antitrypsin (AAT), carcinoembryonic antigen (CEA), macrocreatinine kinase type 2 (M-CK 2; EC 2.7.3.2), "electrophoretically fast, homoarginine-sensitive" alkaline phosphatase (FHAP; EC 3.1.3.1), CK isoenzyme BB, ceruloplasmin, ferritin, beta human chorionic gonadotropin (β-HCG), and salivary-type isomethylase (S-AMY, EC 3.2.1.1) in 33 symptomatic patients with benign and malignant colorectal disease. Each marker was assayed individually and its diagnostic sensitivity and specificity were compared with those of other markers, both singly and in combination.

Materials and Methods

Clinical Specimens

Serum from blood specimens sent by physicians for CEA assay during a six-month period was collected from the Department of Pathology, Montefiore Hospital. All samples were taken from symptomatic patients before diagnosis, and medical records were reviewed to obtain pertinent clinical information. Patients selected for evaluation in this study included 12 with late-stage (Dukes's stage D) colorectal malignancy, seven men and five women, ages 54 to 81 except for one 19-year-old woman; 11 with early-stage colorectal malignancy (modified Dukes's stages A or B), five men and six women, ages 31 to 88; and 10 with benign intestinal disease, three men and seven women, 38 to 81.

Serum samples were assayed immediately or refrigerated at 4°C for several days until analysis.

Control samples were obtained from healthy laboratory personnel.

Procedures

Column-chromatographic separation of isoenzymes. M-CK 2, CK-BB, and FHAP were isolated by a slightly modified version of an ion-exchange column chromatographic procedure previously described for separation of CK isoenzymes (7). We applied 1.0 mL of serum to the top of a 15 × 0.8 cm polyethylene column (Berwick Medical Products, Inc., Horsham, PA 19044) filled to a height of 4.5 cm with DEAE-A50 Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ 08854), and collected the sample effluent in the first fraction. Subsequent fractions were collected after stepwise elution with Tris HCl (50 mmol/L) containing, successively, 100 and 200 mmol of sodium chloride per liter (pH 8.0 at 25°C) and 300 mmol of sodium chloride per liter (pH 7.0 at

1 Nonstandard abbreviations: DEAE, diethylaminoethyl; AAG, α1-acid glycoprotein; CRP, C-reactive protein, AAT, α1-antitrypsin; CEA, carcinoembryonic antigen; FHAP, "fast, homoarginine-sensitive" alkaline phosphatase; CK-BB, brain isoenzyme of creatine kinase; M-CK 2, macro-creatinine kinase (type 2); β-HCG, beta-subunit of human chorionic gonadotropin; S-AMY, salivary isoenzyme of amylase.
25 °C). We collected three fractions of the 100 mmol/L sodium chloride buffer (Fraction 1, 1.0 mL; Fraction 2, 2.0 mL; and Fraction 3, 4.0 mL), four fractions of the 200 mmol/L sodium chloride buffer (Fraction 4, 1.0 mL; Fraction 5, 3.0 mL; Fraction 6, 4.0 mL; and Fraction 7, 4.0 mL), and two fractions of the 300 mmol/L sodium chloride buffer (Fraction 8, 0.5 mL, and Fraction 9, 3.0 mL).

M-CK 2 and CK-BB in the column eluates were identified by electrophoresis on agarose gel (Corning Medical & Scientific, Medfield, MA 02052), and FHAP by electrophoresis on polyacrylamide gel (California Immuno Diagnostics, San Marcos, CA 92069).

S-AMY was isolated by a modified version of a previously reported column procedure (8). We mixed 200 μL of serum with 200 μL of Tris buffer (100 mmol/L, pH 8.6) and applied this to the top of the same kind of polyethylene column as above, but filled to 4.5 cm with DEAE-Sephacel (Pharmacia). After collecting the sample effluent, we eluted the column stepwise with Tris buffer (100 mmol/L, pH 8.6), collecting six 1-mL fractions. We then collected another six 1-mL fractions on elution with the same Tris buffer with an added 300 mmol of sodium chloride per liter. S-AMY was identified in column eluates by electrophoresis on agarose (Corning) according to the procedure of Leclerc and Forest (9).

Measurement of isoenzyme activity. M-CK 2 was eluted from DEAE-Sephadex in the first fraction. However, the MM isoenzyme of CK is also present in this fraction, so we needed to eliminate the CK activity related to MM isoenzyme. We treated the collected fraction with anti-MM-CK antibody from the "Isomune CK" kit (Roche Diagnostics, Nutley, NJ 07110), as follows. Add 200 μL of twofold-diluted goat CK-M antiserum to 400 μL of the MM eluate fraction and incubate for 5 min at room temperature. The CK-M antibodies react with the M-subunit of MM isoenzyme (10) but not with M-CK 2 of mitochondrial origin (11). Now add insoluble second antibody, 100 μL of donkey anti-goat gamma-globulin conjugated to polyvinylidene fluoride flourescures, and incubate at room temperature for 5 min. Centrifuge (1000 × g, 5 min) and remove the insoluble complex formed between polymer–second antibody and CK-M–first antibody. Assay CK activity in the supernate with CK reagent (Beckman Instruments, Carlebad, CA 90208). It is also necessary to assay the supernate for potentially high concentrations of uninhibited adenylate kinase by using CK-blank reagent (Helena Laboratories, Beaumont, TX 77704). Subtract this activity from the total CK activity.

The remaining CK activity is now ascribable to the presence of M-CK 2. Electrophoresis (Corning) of the supernate after electrophoresis with a Centricon micro-concentrator (Amicon Corp., Danvers, MA 01923) revealed a single caged band of M-CK 2 activity close to the point of sample application. Our mean normal value for M-CK 2 in serum from healthy adults (n = 13) is 0.6 (SD 0.3) U/L. The between-run CV was 17.5% (n = 9) at the normal range of activity.

CK-BB was eluted from the DEAE-Sephadex column in Fraction 9. Before assay, we added 10 μL of dithiothreitol (1.5 mmol/L) to 1 mL of Fraction 9 to ensure complete activation of CK-BB, then determined the CK activity in this fraction by using optimized "CK-NAC" reagent (Beckman) as described previously (12), in an Abbott ABA 100 analyzer (Abbott Laboratories, Pasadena, CA 91030). Our mean value for CK-BB in serum of 13 healthy adults was 0.9 (SD 0.2) U/L. The between-run CV for CK-BB was 12.3% (n = 11) for concentrations within the normal range.

FHAP also eluted from the DEAE-Sephadex column in Fraction 9, in a manner similar to the column procedure of Kahan et al. (13). Alkaline phosphatase activity was determined by a kinetic procedure (Beckman) with p-nitrophenyl phosphate as substrate. The mean value for FHAP in serum of 13 healthy adults was 3.2 (SD 1.1) U/L. The between-run CV for FHAP was 8.3% (n = 11) for values within the normal range.

S-AMY was eluted from the DEAE Sephacel column in Fractions 2, 3, and 4 of the second Tris buffer eluent. Amylase activity in these fractions was determined by a kinetic amylose procedure (Beckman) with maltotetraose as substrate. The between-run CV for S-AMY was 12.4% for 10 samples with values within the normal range. The mean value for 13 healthy adults was 30.4 (SD 13.0) U/L.

Measurement of specific proteins. For this we used a kit for homogeneous enzyme immunoassay (xmmr; Syva Co., Palo Alto, CA 94303) of CRP and a Gilford Model S111 spectrophotometer (Gilford Laboratories Inc., Oberlin, OH 44074), used according to Syva specifications. The mean value for CRP in serum of 13 healthy adults was 2 (SD 1) mg/L. The between-run CV for CRP was 5.7% for 12 samples with values within the normal range.

We used rate nephelometry with the Beckman immunochemical system to quantify AAG, AAT, and ceruloplasmin. The between-run CVs (n = 12) for these analytes were 2.4%, 4.7%, and 4.0%, respectively. The mean values for AAG, AAT, and ceruloplasmin in serum of 13 healthy adults were 480 (SD 95), 1481 (SD 236), and 523 (SD 71) mg/L, respectively.

Solid-phase enzyme immunoassay kits (Abbott Diagnostics, North Chicago, IL 60064) were used to quantify the concentrations in serum of ferritin, CEA, and β-HCG. A Quantum spectrophotometer (Abbott Laboratories, North Chicago, IL 60064) was used to measure the absorbance of the test samples. The mean normal values for ferritin, CEA, and β-HCG in 13 healthy adults were 4890 (SD 4170) ng/L, 150 (SD 70) ng/L, and 20 (SD 20) milli-int. units/L, respectively. The between-run CVs for ferritin (n = 10), CEA (n = 11), and β-HCG (n = 12) were 5.7%, 8.4%, and 12.3%, respectively.

Sensitivity and Specificity Scores

The sensitivity of a test, defined here as the ability to identify true-positive cases in patients with colorectal cancer, is calculated by dividing the number of patients with above-normal concentrations of the marker by the total number of patients with colorectal cancer.

The specificity of a test, defined here as the ability to identify true-negative cases in patients without colonic malignancy, is calculated by dividing the number of patients with normal concentrations of the marker by the total number of patients with benign colorectal disease.

Normal Values

The normal upper limit for the concentration of each marker was arbitrarily set on the basis of the results for 13 healthy adult subjects.

Results

Multiple-marker concentrations in 12 patients with late-stage and 11 patients with early-stage colorectal malignancy are shown in Table 1. The highest sensitivity scores for late-stage disease were observed with AAG, AAT, CRP, M-CK 2, and CEA at 83%. The top markers for early-stage disease were AAG and CRP, at 64%. Eight markers (AAG, CEA, CRP, AAT, CK-BB, M-CK 2, FHAP, and ceruloplasmin) for late-stage disease and three markers (AAG, CRP, and AAT) for early-stage disease exhibited 50% or greater sensitivity.
Table 1. Diagnostic Sensitivity of Single Markers in Patients with Colorectal Cancer

<table>
<thead>
<tr>
<th>Marker</th>
<th>Upper limit of normal</th>
<th>Mean (and SD) concn</th>
<th>No. (%) with above-normal concn</th>
<th>Mean (and SD) concn</th>
<th>No. (%) with above-normal concn</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAG, mg/dL</td>
<td>77</td>
<td>123 (52)</td>
<td>10 (83)</td>
<td>89 (34)</td>
<td>7 (64)</td>
</tr>
<tr>
<td>CEA, ng/mL</td>
<td>3.6</td>
<td>602 (1613)</td>
<td>10 (83)</td>
<td>2.4 (1.0)</td>
<td>2 (18)</td>
</tr>
<tr>
<td>AAT, mg/dL</td>
<td>219</td>
<td>282 (96)</td>
<td>10 (83)</td>
<td>220 (66)</td>
<td>6 (55)</td>
</tr>
<tr>
<td>CRP, mg/dL</td>
<td>0.5</td>
<td>9.3 (8.3)</td>
<td>10 (83)</td>
<td>3.2 (3.7)</td>
<td>7 (64)</td>
</tr>
<tr>
<td>M-CK 2, U/L</td>
<td>1.5</td>
<td>7.1 (8.6)</td>
<td>10 (83)</td>
<td>0.8 (0.6)</td>
<td>3 (27)</td>
</tr>
<tr>
<td>FHP, U/L</td>
<td>6.5</td>
<td>31.1 (40.2)</td>
<td>7 (58)</td>
<td>5.4 (2.8)</td>
<td>3 (27)</td>
</tr>
<tr>
<td>CK-BB, U/L</td>
<td>1.5</td>
<td>4.9 (10.9)</td>
<td>7 (58)</td>
<td>0.8 (0.4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ceruloplasmin, mg/dL</td>
<td>45</td>
<td>46 (11)</td>
<td>6 (50)</td>
<td>40 (6)</td>
<td>2 (18)</td>
</tr>
<tr>
<td>Ferritin, ng/mL</td>
<td>175</td>
<td>212 (214)</td>
<td>4 (33)</td>
<td>110 (118)</td>
<td>2 (18)</td>
</tr>
<tr>
<td>β-HCG, int. units/L</td>
<td>3.0</td>
<td>6.0 (13.5)</td>
<td>3 (25)</td>
<td>1.5 (1.8)</td>
<td>2 (18)</td>
</tr>
<tr>
<td>S-AMY, U/L</td>
<td>69</td>
<td>38 (21)</td>
<td>1 (8)</td>
<td>36 (15)</td>
<td>2 (18)</td>
</tr>
</tbody>
</table>

Single-marker sensitivity (in descending order) for both early- and late-stage patients (n = 23) is listed in Table 2. Here, AAG and CRP were the highest markers, with a sensitivity of 74%. The lowest marker was S-AMY (13%). Sensitivity scores greater than 50% were exhibited by five markers: AAG, CRP, AAT, M-CK 2, and CEA.

Multiple-marker combinations, in descending order of specificity for 10 patients with benign colorectal disease, are shown in Table 3. The highest specificity scores for benign disease were obtained with five markers (CK-BB, M-CK 2, S-AMY, β-HCG, and CEA). All five markers exhibited 100% specificity. The marker with the lowest specificity score was AAG (80%).

In an attempt to improve sensitivity, we tried using multiple markers, selecting a combination of five markers (CEA, AAG, M-CK 2, CRP, and FHAP) that had high sensitivity scores (Table 2). Quantitative results for these five markers in malignant (early- and late-stage), benign, and control cases are shown in Figure 1. Although AAT also demonstrated increased sensitivity, it was not incorporated into the colon panel because the results were in close agreement with those of AAG and CRP. An assessment of the sensitivity and specificity data obtained by using the colon panel is shown in Table 4. The panel combination with one above-normal marker out of five exhibited the highest sensitivity score (96%); we therefore chose this combination for retrospective analysis of patients' data. Specificity scores were not considered.

Comparing data (Table 5) obtained with the colon panel and with the single marker, CEA, revealed marked improvement in the detection of early-stage carcinoma (net gain, 64%). The gain in sensitivity for patients with late-stage disease was more modest (17%). A comparison of specificity of the colon panel and CEA showed a net loss of 30% when the colon panel was used.

Discussion

Since the introduction of CEA, the usefulness of tumor markers in the management of malignancy has been well documented. The relationship between CEA and colonic carcinoma has been studied most extensively, with the finding that persistently high or increasing concentrations of CEA in serum in the postoperative period are strongly suggestive of residual or metastatic disease (14).

Unfortunately, the hope that CEA or other markers would support early and specific diagnoses of malignancy has not been realized. At present, many colonic neoplasms are detected at a fairly late stage of disease, when curative therapy is less effective. However, many physicians believe that existing therapies would be more effective if malignant colonic lesions could be detected while they are still small and localized.

Our initial objective in this study was to evaluate a battery of markers with the hope of discovering a single marker with greater sensitivity for early-stage colorectal malignancy than CEA has. As shown in Table 2, four markers (AAG, CRP, AAT, and M-CK 2) did score higher than CEA, but their sensitivity scores were far from ideal. Failure to find the ideal marker in our group of 11 was not unexpected: the discovery of a universally useful cancer marker appears unlikely, given the biological variability and biochemical diversity of cancer cells (15). Nevertheless, we grouped a panel of highly sensitive colon markers as an alternative approach to the use of a single marker.

The five markers we selected—two isoenzymes (M-CK 2 and FHAP), two acute-phase reactants (AAG and CRP), and one oncofetal antigen (CEA)—had individual sensitivity scores greater than 40% in patients with a diagnosis of
computer program to analyze the sensitivity and specificity of thousands of possible marker combinations and will use it as soon as enough data have been collected for proper statistical analysis.

As shown in Table 4, multiple marker analysis with the colon panel resulted in 96% sensitivity for detecting colorectal cancer when the concentration of any one of the five markers was above normal. The highest detection rate achieved by using a single marker was only 74% (Table 2); therefore, the combined marker approach almost completely compensated for the lack of a universal single marker. This reinforces the view that marker appearance in the sera of patients with colon cancer can be variable, and that various single markers such as CEA may or may not be produced.

The two markers most frequently increased were the acute-phase reactants, AAG and CRP. Other reports (17-19) have also shown AAG and CRP to be excellent indicators of malignancy in the colon and other tissues. Panel markers CEA, M-CK 2, and FHAP also exhibited results that were in close agreement with previous reports (14, 20, 21).

Retrospective comparison with CEA concentrations in patients with early-stage disease revealed that use of the colon panel markedly increased sensitivity, by 64% (Table 5), primarily because of above-normal concentrations of AAG and CRP in patients with concentrations of CEA within the normal range. Thus, AAG and CRP may provide useful probes for monitoring therapy and detecting early recurrence when CEA or other markers are present in normal concentrations. However, their use as sensitive tumor markers requires careful interpretation because benign forms of neocrosis and tissue inflammation can also give rise to abnormal concentrations of these proteins. Use of the colon panel in patients with late-stage colorectal cancer did not appear to offer any significant advantage over the use of CEA alone, except in a few patients in whom CEA concentrations remained within normal limits.

Thus, the preliminary studies presented here demon-
strate several advantages of the multiple-marker approach: a broader data-base, leading to increased diagnostic sensitivity, and the opportunity to find other markers for detecting early recurrence and monitoring therapy.

The major disadvantage of the use of multiple markers is related to the nonspecific nature of the markers in current use. Unfortunately, including several such markers in a panel tends to magnify this specificity problem. Here, the colon panel demonstrated only 70% specificity. However, we are confident that specificity can be improved through the use of serial measurements. For example, steadily rising concentrations of markers over time usually reflect increasing tumor burden, while decreasing concentrations indicate decreasing tumor burden. However, a transient increase in a marker or minor fluctuations would most likely be associated with benign disease or with biological variation within the individual patient.

Recently, Cohen and Dix (22) also discussed serial testing as a way to improve sensitivity and specificity for assays of prostatic acid phosphatase. They recommended the establishment of more-accurate intra-individual normal ranges for markers through serial testing. This approach to normal values would make it easier to interpret slight increases in the concentrations of a marker in a given patient because the patient's own normal value would form the basis of comparison. Winkel et al. (16) have also recommended the use of statistical time-series analysis when evaluating cumulative data, in order to eliminate inter-subject variability. Thus, properly analyzed, serial results may greatly enhance the practical evaluation of tumor status.

In our laboratory, we have developed a microcomputer-based system for cumulative reporting of panel results that greatly aids the physician in the clinical management of malignancy. In addition, a computerized recall system, designed to assist the physician in the collection of serial samples, has been developed. Many more studies are required to collect the necessary data needed to properly evaluate the clinical value of serial panel-marker testing. While such studies are in progress, isolated case reports (unpublished) and preliminary clinical studies, as described here, suggest the potential usefulness of panel testing.

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References