Free DNA and Carcinoembryonic Antigen Serum Levels: An Important Combination for Diagnosis of Colorectal Cancer

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Abstract

Purpose: The identification of new molecular markers for the early detection of colorectal cancer has become an important objective. We compared the sensitivity and specificity of free circulating DNA with that of the more conventional carcinoembryonic antigen (CEA) and evaluated the two markers in combination.

Experimental Design: The study was carried out on 75 healthy donors and 75 colorectal cancer patients. Free DNA was determined in serum with quantitative PCR analysis. The diagnostic accuracy of each assay was calculated using receiver operating characteristic (ROC) curves. The diagnostic relevance of the two-marker combination was analyzed by the logistic regression model.

Results: Median free DNA concentration was ~5-fold higher in patients than in healthy donors ($P < 0.001$). The area under the ROC curve was 0.86, and when 12.5 ng/mL was used as cutoff, 81.3% sensitivity and 73.3% specificity were observed for the overall series. As CEA and free DNA provided independent diagnostic information, they were also considered in combination. ROC curve analysis of the combined CEA and free DNA algorithms showed a higher diagnostic capacity (area under the ROC curve, 0.92) than that of markers considered singly, with 84% sensitivity and 88% specificity.

Conclusions: Free circulating DNA, especially when used in combination with CEA, represents a potentially useful tool for the diagnosis of early-stage colorectal cancer.

Worldwide, colorectal cancer has the third and fourth highest incidence and the fifth and fourth highest mortality in females and males, respectively (1). Despite advances made, the efficacy of therapy has reached a plateau, making early diagnosis fundamental to reduce morbidity and mortality, especially as it is known that patients diagnosed at early stages show long-term survival (2).

The most widely used screening technique for colorectal cancer is the fecal occult blood test. However, this simple, inexpensive, and noninvasive test is heavily prone to produce not only false-positive results but also false-negative results because colorectal tumors bleed intermittently.

On the other hand, colonoscopy, which has very high diagnostic accuracy in terms of both sensitivity and specificity, is characterized by a moderate compliance because it is invasive and not without potentially adverse events. Its use is limited to second-level diagnostic tests within screening programs (3–5).

Numerous serum markers, such as carcinoembryonic antigen (CEA), carbohydrate antigen 19-9, and lipid-associated sialic acid, have been investigated in colorectal cancer, but their low sensitivity has induced the American Society of Clinical Oncology to state that none can be recommended for screening and diagnosis and that their use should be limited to postsurgery surveillance (2, 4, 6–8).

The search, therefore, continues for markers that match the diagnostic accuracy of fecal occult blood test but are less invasive than colonoscopy for the early detection of colon cancer.

Free circulating DNA has produced interesting results for lung and breast cancer (9–12). The few studies of this marker done for colon cancer have shown higher levels of circulating DNA in patients than in healthy individuals (13–15), but its diagnostic relevance has not been adequately investigated. The biological characterization of circulating DNA in the blood of patients has shown that an important component derives from tumor cells (16, 17). Although the mechanism of DNA release into the blood is unknown, cell apoptosis or necrosis, as well as the active release of DNA, have been hypothesized (18).

The aim of the present study was to define the relevance of a relatively simple and inexpensive test based on circulating DNA alone and in combination with the more conventional CEA analysis (6–8) for the early detection of colorectal cancer.

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Materials and Methods

Case series

This case-control study comprised 75 healthy donors and 75 colorectal cancer patients. Patients at first diagnosis of colorectal cancer and not previously treated with any type of therapy were recruited from the Departments of General Surgery I and II of Morgagni-Pierantoni Hospital (Forlì, Italy), from December 2003 to February 2005. Healthy donors were enrolled during the same period from the Blood Transfusion Service and the Department of General Surgery I of the same hospital, and none had at that time or had previously had any type of cancer.

The median age of healthy individuals was 57 years (range, 29-79 years) and that of patients was 71 years (range, 38-89 years). The control group comprised 27 females and 48 males, whereas 32 females and 43 males made up the patient group.

Twenty-two cancers were located in the right colon, 38 in left colon, 3 in the transverse colon, and 12 in the rectal tract. Tumors were classified according to Dukes' staging system: 18 were stage A, 19 were stage B, 26 were stage C, and 12 were stage D.

The study protocol was reviewed and approved by the local ethics committee in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All subjects gave their informed written consent to take part.

Sample collection

A 5-mL venous blood sample from patients presurgery and from controls was collected in tubes without anticoagulant and centrifuged at 2,500 rpm for 15 minutes at room temperature. Serum was then stored at −80°C until processing.

Marker determination

Carcinoembryonic antigen. The CEA assay was done on serum in the Clinical Pathology Laboratory of Morgagni-Pierantoni Hospital using chemiluminescent microparticle immunoassay technology (Abbott Laboratories, Abbott Park, IL). The Architect CEA assay consists of a two-step quantitative immunoassay. The limit of normality was considered as 5 ng/mL.

Free DNA. Serum samples were processed within a maximum of 2 months to avoid biases caused by prolonged sample storage. DNA extracted from 1 mL serum was purified according to the protocol used by Sozzi et al. (9, 10) and stored at −20°C.

Table 1. CEA levels in serum

	Healthy donors		Patients	
	n	Median (range), ng/mL	*n*	Median (range), ng/mL
All	75	1.5 (0.5-6.9)	75	3.8 (0.8-1,483.8)
Gender				
F	27	1.3 (0.5-4.2)	32	4 (0.8-633.2)
M	48	1.8 (0.5-6.9)	43	3.6 (1-1,483.8)
Age (y)				
<60	50	1.5 (0.5-6.9)	14	2.3 (0.9-303.3)
≥60	25	1.7 (0.5-3.9)	61	4 (0.8-1,483.8)
Tumor site				
Right			22	2.5 (0.9-266.3)
Left			38	4.1 (0.8-1,483.8)
Transverse			3	9.9 (4-57.7)
Rectal			12	7.3 (1-633.2)
Dukes' stage				
A			18	2.4 (0.8-14.2)
B			19	4.2 (0.9-303.3)
C			26	3.7 (1.5-633.2)
D			12	20.6 (1.4-1,483.8)

Table 2. Free circulating DNA in serum

	Healthy donors		Patients	
	n	Median (range), ng/mL	*n*	Median (range), ng/mL
All	75	7.7 (0.2-46.6)	75	35.8 (0.6-153)
Gender				
F	27	6.5 (0.2-27.9)	32	42.5 (0.6-153)
M	48	8 (1.5-46.6)	43	30 (3.6-100)
Age (y)				
<60	50	8.4 (0.2-27.9)	14	33 (2.5-121)
≥60	25	6.9 (0.3-46.6)	61	35.8 (0.6-153)
Tumor site				
Right			22	35.6 (5.1-153)
Left			38	27.1 (2.5-141)
Transverse			3	36 (3.6-145)
Rectal			12	39.3 (0.6-64.4)
Dukes' stage				
A			18	37.5 (5.1-153)
B			19	41.7 (3.6-145)
C			26	22.4 (0.6-80.2)
D			12	47.1 (13.8-121)

To quantify circulating free DNA, the *glyceraldehyde-3-phosphate dehydrogenase* housekeeping gene, used recently in a study on lung cancer (11), was amplified by a real-time quantitative PCR assay (19) using SYBR Green I Dye Chemistry (20, 21) and MyIQ Single-Color Real-time PCR Detection System (Bio-Rad, Hercules, CA). Forward and reverse primer sequences were 5′-ACCCAGAAGACTGTGGATGG-3′ and 5′-TTCAGCTCAGGGATGACCTT-3′, respectively. PCR mix was prepared in a total volume of 25 μL containing 1× SYBR Green Supermix (Bio-Rad), 0.4 μmol/L of each primer, and 5 μL DNA. PCR conditions were set up as follows: a first denaturation at 95°C for 3 minutes and 40 cycles for 15 seconds at 95°C and 30 seconds at 55°C for annealing and extension.

The absolute concentration of target DNA was calculated on a standard curve using concentrations ranging from 25 to 0.01 ng DNA extracted from the peripheral blood of a healthy donor. Each sample was run in triplicate and the intra-assay variability was assessed by computing the coefficient of variation among the three C_t values (defined as the fractional cycle number at which the emitted fluorescence exceeds a fixed threshold value above the baseline), which was always <1.5%. The interassay variability between two independent experiments was assessed by the coefficient of variation and was always <15%.

Statistical analysis

Using the best cutoff value, the population size was defined based on an expected sensitivity and specificity of ~75%. For 75 healthy donors and 75 cancer patients, we predicted the 95% confidence intervals (95% CI) to be ±10% with respect to the single estimated value for sensitivity and specificity.

The median values of each marker in controls and cases were compared using the median test. The correlation between serum levels of the two markers was assessed with the Spearman rank test. In the absence of internationally available cutoff values for serum free DNA, the cutoff maximally discriminating between healthy donors and patients was identified using receiver operating characteristic (ROC) curve analysis (22). The 95% CI values were calculated for sensitivity and specificity values. The independent diagnostic relevance of the two markers considered as continuous variables (subjected to natural logarithmic transformation) was analyzed by the logistic regression model (23).

The linear predictor or logit resulting from the model was used as a new diagnostic test on which the ROC curve was calculated. Statistical analysis was done using SPSS software.

Results

Median CEA concentrations were 1.5 ng/mL (range, 0.5-6.9 ng/mL) in healthy donors and 3.8 ng/mL (range, 0.8-1,483.8 ng/mL) in patients ($P < 0.001$). Median free DNA concentrations were ~5-fold lower in healthy individuals (7.7 ng/mL; range, 0.2-46.6 ng/mL) than in patients (35.8 ng/mL; range, 0.6-153 ng/mL; $P < 0.001$). Median concentrations of the two markers were independent of age and gender in both donors and patients and did not differ as a function of clinical or pathologic features, with the exception of Dukes' stage (data not shown). In fact, median CEA levels were similar in Dukes' stage A to C tumors and increased in patients with stage D disease. The lack of an increase from stages B to C could be due to the higher percentage of grade 3 tumors in stage C (31%) compared with stage B (11%). Conversely, free DNA median levels were already high in patients with early-stage tumors (Tables 1 and 2).

The diagnostic accuracy of CEA and free DNA was evaluated using continuous values in ROC curve analysis. The area under the ROC curve (AUC) was 0.82 (95% CI, 0.75-0.89) for CEA and 0.86 (95% CI, 0.80-0.92) for free DNA (Fig. 1).

Sensitivity and specificity were calculated for different cutoff values using the standard 5 ng/mL for CEA and various cutoff values for free DNA (Table 3). Sensitivity and specificity for CEA were 38.7% (95% CI, 28.4-50.0) and 97.3% (95% CI, 90.8-99.2), respectively.

For free DNA levels, sensitivity ranged from 70.7% to 90.7% and specificity varied from 53.3% to 93.3% for the different cutoff values. Specifically, when 12.5 ng/mL were used as the cutoff value, 81.3% (95% CI, 71.0-88.5) sensitivity and 73.3% (95% CI, 62.3-82.0) specificity were observed for the overall series.

As CEA and free DNA were not significantly correlated in either donors ($r_s = 0.04$; $P = 0.77$) or patients ($r_s = 0.16$; $P = 0.17$), the two markers were analyzed simultaneously. When considered as dichotomous variables, positivity to at least one of the two markers resulted in specificity (70.7%; 95% CI, 59.7-79.5) similar to that of a single marker, whereas sensitivity increased (88%; 95% CI, 78.7-93.5). Furthermore, when both were positive, specificity reached 100%. When the markers were used as continuous variables, transformed into logarithmic values, and analyzed in a logistic regression model, each marker provided independent and therefore additive diagnostic information. From the model, a unit increase in log serum DNA or in log CEA was associated with an increase of ~5-fold in cancer risk (Table 4).The ROC curve analysis of the "combined" CEA and free DNA algorithms showed a higher diagnostic capacity than that of either marker considered singly (AUC, 0.92; 95% CI, 0.88-0.92; Fig. 1). The best cutpoint of this algorithm (0.18) was associated with 84% sensitivity and 88% specificity. Sensitivity of the two markers tested in combination was similar in patients with Dukes' stage A (83.3%; 95% CI, 60.4-93.9), B (89.5%; 95% CI, 68.3-96.8), and C (84.6%; 95% CI, 66.3-93.7) and reached 100% in patients with Dukes' stage D disease.

Table 3. Diagnostic relevance of serum CEA and free circulating DNA

Marker	Cutoff, ng/mL	% Sensitivity (95% CI)	% Specificity (95% CI)
CEA	5	38.7 (28.4-50.0)	97.3 (90.8-99.2)
Free DNA	8.5	90.7 (81.9-95.3)	53.3 (42.1-64.2)
	12.5	81.3 (71.0-88.5)	73.3 (62.3-82.0)
	16.5	73.3 (62.3-82.0)	81.3 (71.0-88.5)
	20.5	70.7 (59.5-79.8)	93.3 (85.3-97.0)

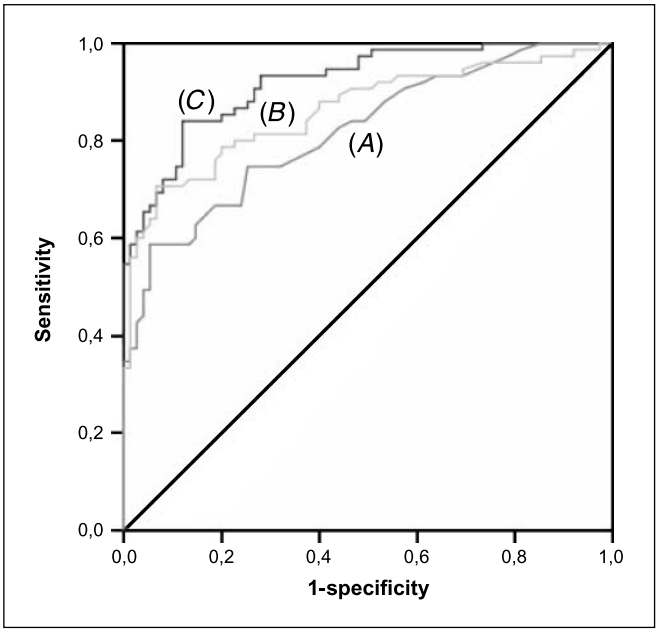

Fig. 1. ROC curves for the diagnosis of colorectal cancer using CEA (*A*), DNA (*B*), and combined CEA and DNA (*C*).

Discussion

For colorectal cancer, large-scale screening programs comparable with those used for breast cancer do not exist, possibly because of poor understanding of screening benefits or as a result of the invasiveness of standard tests (i.e., colonoscopy and sigmoidoscopy; refs. 2, 3).

CEA, the most widely used serum marker in colorectal cancer, offers a sensitivity of only 30% to 40% for early-stage tumors (6–8), also confirmed in our study. A large number of studies have reported higher concentrations of serum/plasma free DNA in patients with various types of cancer (9–12, 24). Seventy-eight percent sensitivity and 95% specificity for circulating free DNA levels have been reported for non–small cell lung cancer patients (10). In patients with breast cancer, higher free DNA levels have been observed compared with those of controls, independently of tumor size and stage (12). In the present study, median CEA levels were lower in patients with Dukes' stage A to C disease and increased in metastatic cancer, whereas high free DNA levels were observed starting from early-stage tumors.

As far as we know, ours is the first case-control study on colorectal cancer patients. Free DNA levels were ~5-fold higher in the serum of patients than in that of controls and were not related to either age or gender. Moreover, circulating DNA showed 81.3% sensitivity, which increased to 88% when free DNA and CEA were considered in association. The combination of the two markers could therefore be a useful tool for the diagnosis of early-stage disease. Based on these findings, the free DNA test would seem to represent a rapid and relatively inexpensive alternative to the more invasive colonoscopy.

Table 4. Combined analysis of the two markers considered as continuous variables in logistic regression model

Variable	Coefficient	SE	Wald test	Odds ratio (95% CI)	*P*
ln CEA	1.671	0.394	17.986	5.317 (2.456-11.509)	<0.001
ln DNA	1.726	0.338	26.073	5.620 (2.897-10.901)	<0.001
Constant	−6.005	1.035	33.696	0.002	

NOTE: logit = −6.005 + 1.726 ln DNA + 1.671 ln CEA.

A comparative analysis of circulating free DNA and DNA amplification in feces, routinely carried out in our laboratory, is currently being done on the same case series to define the diagnostic accuracy of the single tests (25–27).

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