

Prognostic Significance of Circulating Antibodies Against Carcinoembryonic Antigen (Anti-CEA) in Patients With Colon Cancer

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OBJECTIVE: The discovery of antibodies against carcinoembryonic antigen (CEA) in patients with digestive cancers, in the late 1970s, initiated a number of studies on the role of these antibodies in patients with cancers of the GI tract. Our aim was to determine the prevalence and prognostic significance of the IgG and IgM anti-CEA antibodies in the serum of patients with colon cancer.

METHODS: Using an enzyme-linked immunoassay, the sera of 58 colon cancer patients were examined for the presence of carcinoembryonic antigen (CEA) and for circulating antibodies against the CEA (anti-CEA). An inhibition assay was carried out for the determination of the specificity of the IgG and IgM anti-CEA antibodies.

RESULTS: The CEA was elevated (≥ 10 ng/ml) in only 12 patients (20.6%). Anti-CEA IgM and/or IgG antibodies were detected in 46 patients with colon cancer (79.1%). In the control group ($n = 28$), 10% of the individuals had detectable amounts of IgG and/or IgM anti-CEA antibodies. Patients with detectable amounts of circulating IgM anti-CEA antibodies ($n = 14$, 30.5%) had a statistically significantly better 2-yr survival compared to the rest of the patients ($p = 0.017$). The IgM anti-CEA antibodies can also be used as an independent prognostic factor in these patients ($p = 0.0323$).

CONCLUSIONS: In this study, a high number of colon cancer patients have circulating anti-CEA antibodies in their sera. These may be used as diagnostic markers and as independent prognostic factors. In addition, the presence of these antibodies in the patients studied is associated with better prognosis and significantly increased 2-yr survival. It was also found that the anti-CEA antibodies (IgG and IgM) are more sensitive markers than CEA. These findings underline the biological importance of the anti-CEA antibodies and provide additional information on their potential use as markers of the immune status in patients with colon cancer. (Am J Gastroenterol 2000;95:1056–1061. © 2000 by Am. Coll. of Gastroenterology)

INTRODUCTION

Carcinoembryonic antigen (CEA) is a glycoprotein that was initially isolated by Gold and Freedman in 1965 (1). Its molecular weight is 180,000 D. It has a high carbohydrate content (~ 40 –75%) and contains multiple immunoglobulin domains (2). CEA is a cell adhesion molecule and a member of a large, closely related family of molecules sharing many common epitopes (3, 4). CEA is capable of binding to an 80-Kb Kupffer cell receptor by the peptide sequence PELPK, and stimulates cytokine production. Cytokines induce sinusoidal endothelial cells to express intercellular adhesion molecules and increase adhesion of the tumor cells (5). Recent studies in rodents have shown that CEA may be directly involved in the malignant transformation process by blocking the terminal differentiation in rodent myoblasts *in vitro*. In addition, tumor-released CEA is believed to be one of the factors involved in cancer metastases (6). To avoid the effects of the excessive CEA concentration observed in patients with certain malignancies, a number of studies have been designed that aimed to minimize the CEA concentration in the blood circulation. Trials up to now have concentrated on the chemical synthesis of antibodies, or part of antibodies (mABs or Fabs), that would be able to recognize and destroy the CEA molecules in the circulation and, therefore, prevent the spreading of the tumor cells (6–8).

CEA is over-expressed in a wide variety of human malignancies, including colon cancer (9, 10). Elevated CEA levels have also been reported in a small percentage of patients with nonmalignant diseases of the colon, liver, pancreas, and lungs (11, 12). Although a large number of patients with cancer of the large bowel have elevated serum CEA levels, the serum CEA levels may be normal in many colon cancer patients, especially in the early stages of disease (12–15).

Until recently, it was not known whether CEA can evoke an antibody response in the tumor bearing host. In 1967, Gold (16) mentioned the existence of circulating antibodies against CEA in patients with digestive system cancers and in pregnant women. This was challenged by a number of

researchers who claimed that these anti-CEA antibodies are actually anti-blood group antibodies or antibodies of low specificity (12, 16–18). Recently, however, the presence of anti-CEA antibodies has been described in a large number of colon cancer patients. These antibodies were found to be of high specificity and to have a high titer in the sera of cancer patients, although their prognostic significance remained unknown (17, 18).

The major aims of this study were: 1) to study the prevalence of IgG and/or IgM anti-CEA antibodies in patients with colon cancer, and 2) to determine whether the presence of circulating anti-CEA antibodies is associated with a better survival.

MATERIALS AND METHODS

Patients

This was a prospective study conducted in the First Department of Propedeutic Surgery, of the Athens Medical School, Athens, Greece. Sera were preoperatively obtained from 58 consecutive patients (28 female and 30 male) with histologically confirmed colon cancer, before the administration of any type of treatment. The mean age of the patients was 68 yr and the median was 68.5 yr, indicating a normal distribution. The minimum age observed in the group examined was 35 yr and the maximum 93 yr. The follow-up time was ≥ 2 yr.

Among those patients, eight (13.76%) were Dukes stage A, 22 (37.84%) stage B, 24 (41.28%) stage C, and four (6.9%) stage D. All patients had a complete surgical removal of the primary tumor. Stage C patients received standard adjuvant chemotherapy, whereas stage D patients received therapeutic chemotherapy. In both cases, a 5-fluorouracil and leucovorin regimen was administered to these patients.

As negative controls, we used the sera of 28 healthy blood donors (16 male and 12 female) aged 37–79 yr, with a median age of 67 yr. Of the 28 blood donors, 17 were smokers. Each serum sample was divided into aliquots and stored at -20°C until tested.

Methods

Isolation and purification of human CEA was obtained from metastatic colon cancer tumors, following the same procedure described by Konstadoulakis *et al.* (19). The purified CEA preparation led to the isolation of a protein with molecular weight of 180,000 D, which was completely free of contamination.

Determination of the CEA was performed using an ELISA method kit (Abbott, North Chicago, IL) (19). The detection of IgG and IgM anti-CEA antibodies in the sera of the patients was done by performing an enzyme-linked immunoassay (ELISA) specially modified for the detection of anti-CEA antibodies. This method has been previously described by Konstadoulakis and Burtin (18, 19). Briefly, 96-well, flat-bottom microplates (NUNC, Roskilde, Den-

mark) were coated with 0.5 mg/100 ml CEA preparation (5 μg of CEA/well) and incubated for 1 h at 37°C . The plates were then washed and incubated for 1 h at 37°C with 100 μl /well of a 3% phosphate-buffered saline (PBS)–bovine serum albumin (BSA) solution, to prevent nonspecific binding. After further washing of the plates with a $1 \times$ PBS, BSA 1%, TWEEN 0.5% solution, 100 μl of the sera of the patients was added per well at a 1/100 dilution and incubated at 37°C for 1 h. After washing the plates, 100 μl of a peroxidase-labeled antihuman IgG and IgM immunoglobulin (Sigma, St. Louis, MO) was added per well at a dilution 1/1000 for 1 h at 37°C . Detection of the conjugated IgG or IgM anti-CEA antibodies was done with the peroxidase method using O-phenylenediamine as the substrate for the production of a yellow color. The reaction was terminated with concentrated hydrochloric acid (0.1 N). The enzymatic activity was then examined by immediately measuring the absorbance at 492 nm. For the formation of the curve for the estimation of the concentration of IgG and IgM anti-CEA antibodies in the sera of the patients, different dilutions of neat IgG and IgM, varying from 1/10 to 1/50, were used.

To study the specificity of the anti-CEA antibodies, an inhibition assay was used. Sera samples of the patients at 1/50 dilution were preincubated at 37°C for 1 h with a series of dilutions of pure antigen (concentrations 0–80 $\mu\text{g}/\text{ml}$). These sera were then added to an ELISA plate coated with CEA (5 $\mu\text{g}/\text{well}$). The subsequent steps were the same as those described above for the ELISA method. Results in this case were expressed as inhibition of the total anti-CEA activity. In both cases (ELISA and the inhibition assay), a 3% bovine serum albumin (BSA) solution was used as the negative control.

Statistical Analysis

Cumulative survival curves were calculated with the Kaplan-Meier method. Univariate analysis comparing the various patient subgroups was performed with the log-rank test. Multivariate analysis was performed using the Cox model of proportional hazards relation to incorporate all the variables that were found significant in the univariate analysis. Values of $p < 0.05$ indicated statistical significance (13).

The parameters that were statistically examined for correlations were the 2-yr survival of patients with IgG and/or IgM anti-CEA antibodies, the elevated CEA levels in the sera of the patients, and the grade and stage of disease. Because only a small number of patients were Dukes stage A and D (eight and four patients, respectively), patients were categorized according to their stage. Two groups were obtained, one with all Dukes A and B patients and one with all Dukes C and D patients.

The χ^2 test was carried out for the determination of statistically significant differences comparing the smoking status, CEA concentration, and presence of anti-CEA antibodies (IgG and/or IgM anti-CEA) between the patients and the control group. For the determination of differences in

Table 1. Presence of CEA and Anti-CEA IgG and/or IgM Antibodies in the Sera of Colon Cancer Patients and Healthy Individuals

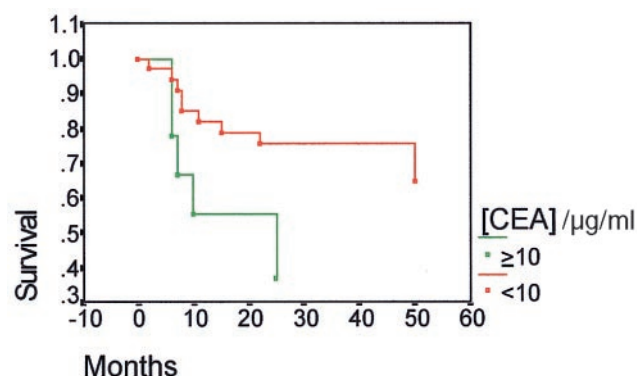
	Patients (n = 58)	Controls (n = 28)	p Value
CEA ≥ 10 ng/ml	12/58 (20.6%)	0/28 (0%)	0.0073
IgG anti-CEA antibodies	14/58 (24%)	1/28 (3.6%)	0.0063
IgM anti-CEA antibodies	13/58 (22.4%)	1/28 (3.6%)	0.0025
IgG and IgM anti- CEA antibodies	19/58 (32.7%)	1/28 (3.6%)	0.00097

age and gender between the two groups, two tests were carried out: the test for proportion for the former, and the Mann-Whitney *U* test for the latter (21). Further χ^2 tests were carried out to determine the association between the presence of IgG and/or the IgM anti-CEA antibodies and age, sex, Dukes stage, grade, and recurrence rate in the group of patients (19, 21).

RESULTS

Of the 58 patients examined, 12 had increased CEA levels in their sera (20.6%) and 46 (79.1%) had detectable amounts of IgG and/or IgM anti-CEA antibodies. Of the 46 patients with CEA concentrations <10 ng/ml, 14 (24%) had detectable IgG anti-CEA antibody levels, 13 (22.4%) had detectable IgM anti-CEA antibody levels, and 19 (32.7%) had both IgG and IgM anti-CEA antibodies in their sera (Table 1).

In the control group (n = 28), CEA concentrations were

**Figure 2.** Comparison of the survival between colon cancer patients with circulating CEA values ≥ 10 $\mu\text{g/ml}$ and <10 $\mu\text{g/ml}$. Patients with low CEA values (<10) had a better 2-yr survival, although not statistically significant.

<10 ng/ml in all cases. Three individuals (10.8%) had detectable amounts of anti-CEA antibodies in their sera. One patient (3.6%) had IgG anti-CEA antibodies, one (3.6%) had IgM anti-CEA antibodies, and one (3.6%) had both (Table 1).

The group of patients and the control group were well matched regarding smoking status ($p = 0.856$), gender ($p = 0.667$), and age ($p = 0.433$). These two groups were significantly different in the CEA concentration (≥ 10 ng/ml) ($p = 0.0073$) and the presence of IgG ($p = 0.0063$), IgM ($p = 0.0025$), or both anti-CEA antibodies ($p = 0.00097$) (Table 1).

Using the χ^2 test it was determined that the presence of IgG and/or IgM anti-CEA antibodies was not significantly

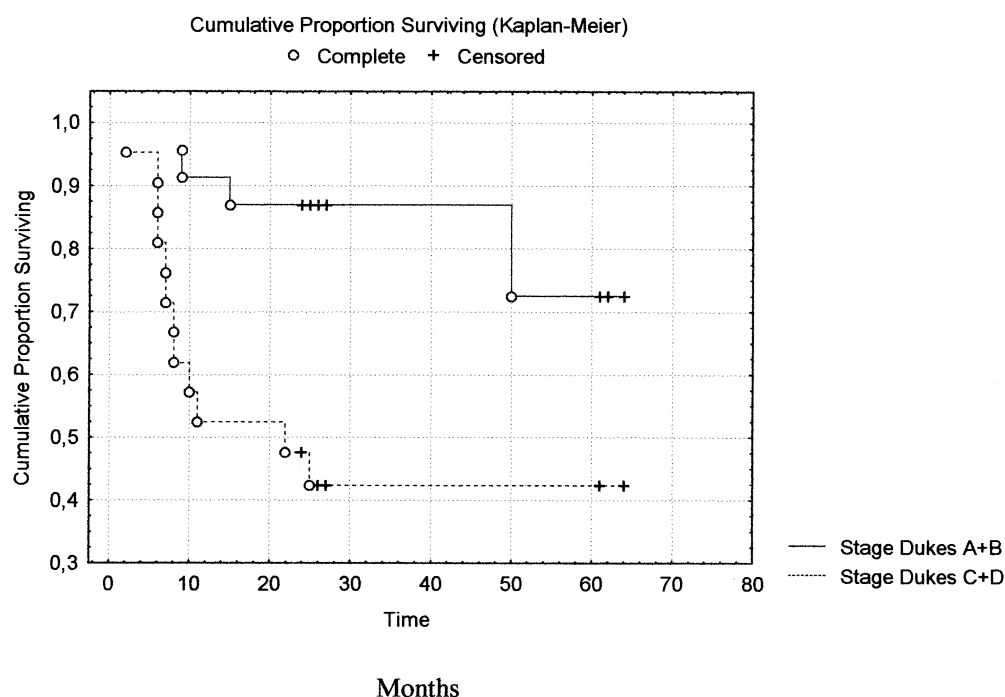
**Figure 1.** Comparison of the survival between patients with different colon cancer stages (Kaplan-Meier). Patients with stage A and B had a better 2-yr survival compared to stage C and D patients.

Table 2. Prognostic Factors in Colon Cancer Patients Examined in This Study

Parameter	Patients	Univariate	Multivariate
CEA			
<10 ng/ml	46	NS	NS
≥10 ng/ml	12		
Sex			
male	33	NS	NS
female	25		
Stage (Dukes)			
A + B	29	$p = 0.0005$	$p = 0.006$
C + D	28		
IgM anti-CEA	13	$p = 0.018$	$p = 0.0323$
IgG anti-CEA	14	$p = 0.057$ (NS)	NS
IgM and IgG anti-CEA	19	NS	NS

NS = not significant ($p > 0.05$).

associated with age, Dukes stage, or grade; in each case, $p > 0.05$.

Survival

From the univariate analysis, Dukes stage ($p = 0.0005$, Fig. 1) and the presence of IgM anti-CEA levels ($p = 0.018$, Fig. 2) proved to be the only statistically significant prognostic factors (Table 2). With regard to CEA concentration, it was determined in this study that CEA cannot be used as a prognostic factor in colon cancer patients ($p = 0.069$).

Patients with detectable amounts of IgM anti-CEA antibodies in their sera had a better 2-yr survival compared to those who were negative (56.5% vs 43.75%, $p = 0.018$, Fig. 3). Although patients with IgG anti-CEA antibodies in their sera also had a better 2-yr survival compared to the rest of

the patients, this difference was not statistically significant (76% vs 50% $p = 0.06$, Fig. 4).

Multivariate statistical analysis was also carried out for every factor examined in this study. It was determined that the only independent prognostic factors were the stage ($p = 0.006$) and the existence of IgM anti-CEA antibodies ($p = 0.0323$).

DISCUSSION

Elevated blood CEA concentrations have been reported in a number of different neoplasms, particularly those of the GI system (10, 13, 14). Although CEA is valid tumor marker for the follow-up of colon cancer patients, its use in diagnosis is limited because of its frequent elevation in a number of nonneoplastic disorders (10, 13). In addition, there is a significant number of colon cancer patients with no detectable blood CEA levels (13, 15, 17). For this reason we examined the prevalence and prognostic significance of circulating IgG and IgM anti-CEA antibodies in the colon cancer patients.

Of the 58 colon cancer patients examined, a large number (46) had detectable amounts of circulating IgG and or IgM anti-CEA antibodies in their sera. The presence of IgG anti-CEA antibodies did not statistically correlate with better survival ($p = 0.057$). However, from the Kaplan-Meier curve it can be seen that patients with anti-CEA IgG antibodies in their sera had a better survival after the first 8 months of the follow-up compared to those patients who were anti-CEA negative (Fig. 2). Hence, there is always the possibility of achieving a statistically significant difference

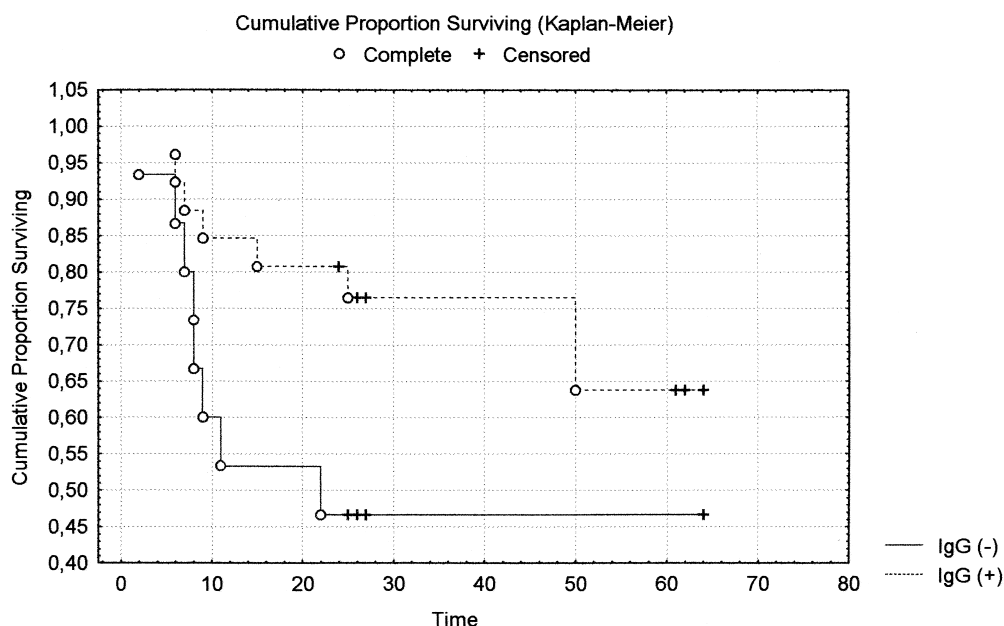


Figure 3. Comparison of the survival between colon cancer patients, with and without circulating IgG anti-CEA antibodies. It can be seen that after the first 8 months of the follow-up, patients with detectable amounts of IgG anti-CEA antibody in their sera had a better survival compared to the rest of the group.

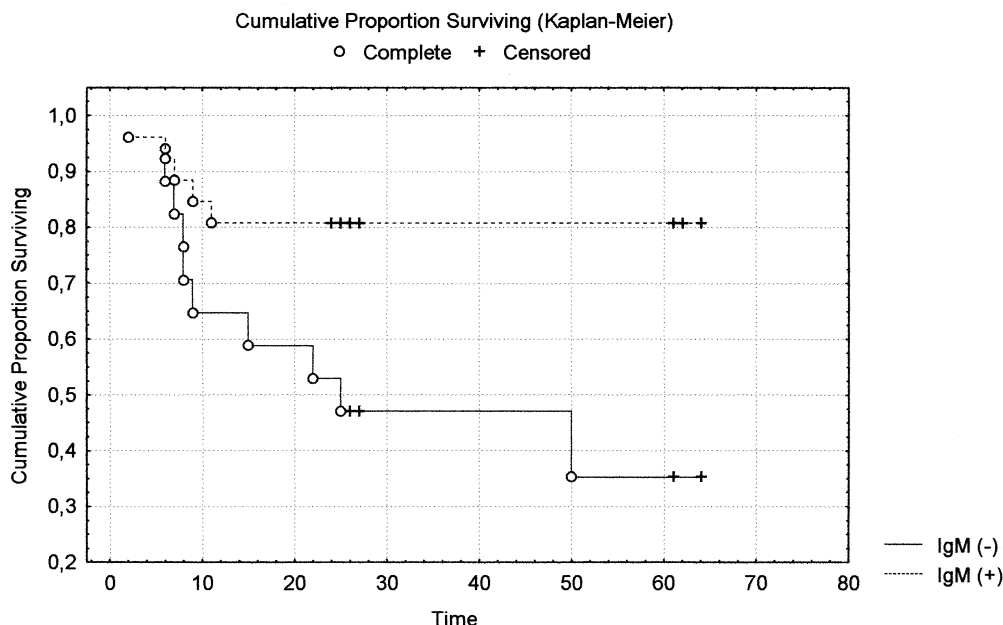


Figure 4. Comparison of the survival between colon cancer patients, with and without circulating IgM anti-CEA antibodies. Patients with detectable amounts of IgM anti-CEA antibodies had a statistically significant better 2-yr survival.

if a larger number of patients is examined and followed-up for a longer period.

Patients with IgM anti-CEA antibodies in their sera, had a statistically significant better 2-yr survival, compared with the rest of the patients ($p = 0.017$, Fig. 1). In the multivariate statistical analysis, it was determined that IgM anti-CEA antibodies ($p = 0.0323$), together with stage ($p = 0.0005$) were the only independent prognostic factors in our patient group. This indicates that these antibodies may represent a useful marker of the immune response of the patient. Patients with a normally functioning immune system produce anti-CEA antibodies and have prolonged survival.

A large number of patients with normal CEA levels had increased anti-CEA IgG and/or IgM antibody concentrations in their sera ($n = 46$, 79.1%). The presence of anti-CEA antibodies in these patients may be due to the lack of release of CEA from the tumor in the circulation, whereas the immune cells are exposed to noncirculating antigens by directly contacting the tumor.

The percentage of the patients with elevated blood CEA concentration in this study was low (21%). According to the literature, almost two-thirds of the patients with colon cancer have elevated CEA levels in their sera. This, however, has been the case in previous studies with colon cancer patients of Greek and some other nationalities, and may be due to genetic variations in the CEA-producing gene (12, 18, 22, 23).

Anti-CEA antibodies were also found present in a small number of healthy individuals (three of 28, 10.5%). This prohibits their use as a conventional tumor marker. Their presence in healthy individuals could be due to their prior antigenic exposure to CEA-releasing malignancies (immunosurveillance).

In summary, in this study we were able to show that the presence of circulating anti-CEA antibodies, especially of the M isotype, in colon cancer patients, correlates with a statistically significant better survival. It was also determined from the multivariate statistical analysis that the IgM anti-CEA antibodies, apart from being associated with better prognosis in patients with colon cancer, can also be used as independent prognostic factors. These findings indicate the biological significance of these antibodies in colon cancer patients.

Further studies are required for the determination of the biological mechanism that leads to the generation of the anti-CEA antibodies in some healthy individuals, and their correlation with other markers of the immune status of cancer patients.

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