

Identification of Cripto-1 as a Novel Serologic Marker for Breast and Colon Cancer

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Abstract **Purpose:** Human Cripto-1 (CR-1), a cell membrane glycosylphosphatidylinositol-anchored glycoprotein that can also be cleaved from the membrane, is expressed at high levels in several different types of human tumors. We evaluated whether CR-1 is present in the plasma of patients with breast and colon cancer, and if it can represent a new biomarker for these malignancies. **Experimental Design:** We determined CR-1 plasma levels using a sandwich-type ELISA in 21 healthy volunteers, 54 patients with breast cancer, 33 patients with colon carcinoma, and 21 patients with benign breast lesions. Immunohistochemical analysis was also used to assess CR-1 expression in cancerous tissues. **Results:** Very low levels of CR-1 (mean \pm SD) were detected in the plasma of healthy volunteers (0.32 ± 0.19 ng/mL). A statistically significant increase in the levels of plasma CR-1 was found in patients with colon carcinoma (4.68 ± 3.5 ng/mL) and in patients with breast carcinoma (2.97 ± 1.48 ng/mL; $P < 0.001$). Although moderate levels of plasma CR-1 were found in women with benign lesions of the breast (1.7 ± 0.99 ng/mL), these levels were significantly lower than in patients with breast cancer ($P < 0.001$). Finally, immunohistochemical analysis and real-time reverse transcription-PCR confirmed strong positivity for CR-1 in colon and/or breast tumor tissues. **Conclusion:** This study suggests that plasma CR-1 might represent a novel biomarker for the detection of breast and colon carcinomas.

Human Cripto-1 (CR-1) is the founding member of the epidermal growth factor (EGF)-CFC family (Cripto in humans, FRL1 in *Xenopus*, and Cryptic in mice) of structurally related proteins that have been identified in several vertebrate species (1–4). The EGF-CFC protein family is characterized by the presence of two important functional domains: a modified

EGF-like domain and a unique cysteine-rich CFC motif. The variant EGF-like motif in the EGF-CFC proteins is divergent from the canonical EGF-domain that is present in EGF-related peptides such as EGF, transforming growth factor α , and heregulins, and explains the observation that CR-1 does not directly interact with type I receptor tyrosine kinases (i.e., EGF-receptor, erbB-2, erbB-3, or erbB-4; refs. 5, 6). The COOH terminus contains, in some cases, consensus sequences for a glycosylphosphatidylinositol anchorage site that serves to attach the protein to the cell membrane. However, CR-1 can also be found in the conditioned medium of several cell lines, probably by cleavage of the glycosylphosphatidylinositol linkage by phosphatidylinositol-phospholipase C or glycosylphosphatidylinositol-phospholipase D, suggesting that EGF-CFC proteins can function as secreted or cell membrane-associated proteins (7–9).

EGF-CFC proteins perform regulatory functions related to cell and tissue patterning during embryogenesis (1, 3, 4). CR-1 has been shown to maintain the pluripotential capacity and self-renewal of human and mouse embryonic stem cells and is therefore considered a stem cell marker (10, 11). In the adult, CR-1 is reexpressed in an inappropriate fashion in a wide range of epithelial cancers, suggesting a link between stem cells and tumor biology (12). In this regard, CR-1 is expressed at elevated levels, as detected by immunohistochemistry, in 50% to 80% of different types of primary human carcinomas, including breast, colon, stomach, pancreas, lung, ovary, cervix, and testis (2, 13). The high frequency of expression of CR-1

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in different types of human carcinomas, and its low expression in normal tissues, suggests that CR-1 may be a suitable target for therapy in human malignancies. In fact, anti-CR-1 antisense oligonucleotides or neutralizing blocking monoclonal antibodies have been shown to strongly inhibit the *in vitro* and *in vivo* growth of human breast, colon, ovarian, and testicular carcinoma cells (14–18). Experiments *in vitro* have shown that CR-1 can increase migration, invasion, epithelial-to-mesenchymal transition, and branching morphogenesis of several mouse and human mammary epithelial cell lines and can promote tumor angiogenesis (2, 19–23). Finally, transgenic mice studies have shown that overexpression of a human CR-1 transgene in the mammary gland under the control of the mouse mammary tumor virus (MMTV) or whey acidic protein promoter results in mammary hyperplasias and adenocarcinomas (19, 24, 25).

In the present study, we investigated whether CR-1, such as other glycosylphosphatidylinositol-linked tumor markers including carcinoembryonic antigen, can be released by tumor cells and reach the bloodstream, where it could be specifically detected for diagnostic and prognostic purposes (26). We therefore developed a sandwich-type ELISA to analyze CR-1 levels in plasma samples from healthy volunteers and from patients with colon and breast cancer. Immunohistochemistry and real-time reverse transcription-PCR (RT-PCR) were used to detect CR-1 expression in colon and/or breast tumor tissues.

Materials and Methods

Plasma from healthy donors and cancer patients. After obtaining informed consent, plasma samples were collected in EDTA-containing vacutubes from a total of 108 patients at ITN-Fondazione Pascale in Naples, Italy. Of these patients, 87 were diagnosed with colon and breast cancer and 21 patients were diagnosed with benign breast lesions (fibrocystic disease, adenosis, and fibrosis). Plasma samples from 21 healthy volunteers were also collected and analyzed as controls. All plasma samples were stored at -80°C until further analysis.

Plasma of MMTV-1 CR-1 transgenic mice. Plasma samples from MMTV-CR-1 transgenic mice and FVB/N control mice were centrifuged at 4°C for 15 minutes at 2,000 rpm and were stored at -80°C until further analysis.

Recombinant proteins and antibodies. Recombinant human CR-1, EGF, amphiregulin, heparin-binding EGF, and heregulin $\beta 1$ were purchased from R&D Systems (Minneapolis, MN). The mouse monoclonal anti-CR-1 antibody (A10.B2.18) was kindly provided by Biogen-Idec (Cambridge, MA). Rabbit polyclonal anti-CR-1 antibody was developed by Biocon (Frederick, MD).

ELISA for CR-1 in plasma samples. A sandwich-type ELISA was developed for the quantitative measurement of soluble CR-1 in plasma samples. The anti-CR-1 monoclonal antibody A10.B2.18 (1 $\mu\text{g}/\text{well}$) was adsorbed to 96-well microtiter plates for 1 hour at room temperature. The plates were blocked with 2% milk, washed thrice with washing buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and incubated with 100 μL of undiluted plasma samples, CR-1 recombinant protein (diluted in 1% milk) at concentrations ranging from 3.7 pg/well (100 μL) to 2 ng/well (100 μL), and negative controls (2% milk blocking buffer) overnight at 4°C . Unbound CR-1 was removed by washing the plates five times with washing buffer (Kirkegaard & Perry Laboratories) and a rabbit polyclonal anti-CR-1 antibody (1:3,000; Biocon) was added to the plates for 1 hour at room temperature. The plates were then washed five times with washing buffer and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:3,000; Amersham Pharmacia Biotech, Piscat-

away, NJ) for 1 hour at room temperature. The plates were developed with 3,3',5,5'-tetramethyl-benzidine peroxidase substrate (Kirkegaard & Perry Laboratories) and absorbance was read at 450 nm. All the samples were assessed in duplicate.

Determination of sensitivity and specificity. Recombinant human CR-1 protein was used to generate a standard curve. Cross-reactivity with other homologous EGF-like proteins was investigated using purified recombinant EGF, amphiregulin, heparin binding-EGF, and heregulin $\beta 1$ at a concentration of 20 $\mu\text{g}/\text{mL}$. The sensitivity of the CR-1 sandwich ELISA was defined as the ratio between true positives (TP) and the sum of true positives and false-negatives (FN) [$\text{TP} / (\text{TP} + \text{FN})$]; specificity was defined as the ratio between true negatives (TN) and the sum of true negatives and false-positives (FP) [$\text{TN} / (\text{TN} + \text{FP})$]. Accuracy was defined as the ratio between the sum of true positives and true negatives and the total number of subjects (n) [$(\text{TP} + \text{TN}) / n$].

Immunohistochemistry. After surgery, matching tumor tissue samples were obtained from 15 patients with breast carcinoma and 7 patients with colon carcinoma, and were used for immunohistochemistry. Each 5- μm paraffin-embedded section was dewaxed in xylene and rehydrated in a series of graded ethanols. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 10 minutes. Antigen retrieval was done by incubating the sections with ready-to-use proteinase K solution (DakoCytomation, Carpinteria, CA) for 6 minutes at room temperature and washed several times in PBS. Immunostaining with anti-CR-1 monoclonal antibody (MAB2771, diluted to 1 $\mu\text{g}/\text{mL}$; R&D Systems) was done using the Vectastain ABC immunoperoxidase kit and Vector 3,3'-diaminobenzidine peroxidase substrate kit (both from Vector Labs, Burlingame, CA) following the manufacturer's instructions. Sections were counterstained with hematoxylin and observed with a light microscope. The intensity of CR-1 expression was evaluated by scoring the percentage of positive staining cells per field observed. The final score per section analyzed was calculated from the mean percentage of staining from at least four different fields observed at $400\times$ magnification.

Real-time RT-PCR. For real-time RT-PCR, normal human breast from reduction mammoplasty, and breast cancer tissue samples were obtained from the Fox Chase Cancer Center. RNA was isolated from these samples as previously described (27). Quantification of CR-1 mRNA was done using TaqMan technology. The following primers and probe for the analysis of CR-1 mRNA expression were selected by using Primer Express software (PE Biosystems, Foster City, CA): forward primer 5'-CACGATGTGCGCAAAGAGA-3'; reverse primer 5'-TGACCGTGCCAGCATTTACA-3'; TaqMan probe [labeled with carboxyfluorescein (FAM)] 5'-CCATGACACCTGGCTGCCCAAGAA-3'. All RT-PCR reactions were done on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the fluorescent TaqMan methodology (TaqMan One-Step RT-PCR Master Mix Reagents, Applied Biosystems). The PCR cycle at which the fluorescence intensity increases above the background signal is called the cycle threshold (Ct). In total, 100 ng of total RNA was used for each RT-PCR reaction in a total volume of 50 μL . Primers and probe concentration for the target gene were optimized according to the manufacturer's procedure. The thermal cycling conditions comprised 30 minutes at 48°C , 10 minutes at 95°C , and 40 cycles of 15-second denaturalization at 95°C and 60-second annealing at 60°C . Each sample was analyzed in triplicate, normalized against 18S as a control gene and was expressed in relation to a calibrator sample. Relative quantification of CR-1 mRNA within the samples examined was done by using the $\Delta\Delta\text{Ct}$ method ($\Delta\text{Ct}_{\text{sample}} - \Delta\text{Ct}_{\text{calibrator}} = \Delta\Delta\text{Ct}$; relative quantity = $2^{-\Delta\Delta\text{Ct}}$), as suggested by the manufacturer (PE Biosystems).

Statistical analysis. The nonparametric Mann-Whitney U test was used to assess the statistical significances of the differences between the various groups. Pearson's regression analysis and Kruskal-Wallis rank sum test were used to assess the correlation between CR-1 values in cancer patients and clinicopathologic variables. Statistical calculations

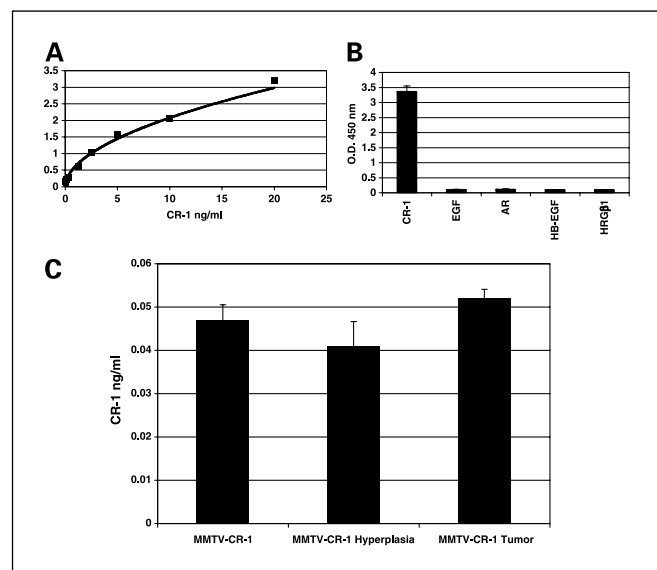


Fig. 1. Sandwich ELISA for human CR-1. *A*, standard curve of human CR-1 recombinant protein diluted to a concentration ranging from 0.037 to 20 ng/mL. *B*, specific detection of CR-1 (20 ng/mL) measured with sandwich ELISA. The EGF-related proteins tested for cross-reactivity were EGF, amphiregulin (AR), heparin binding-EGF (HB-EGF), and heregulin β 1 (HRG β 1; 20 μ g/mL). *C*, sandwich ELISA for CR-1 in the plasma of MMTV-CR-1 transgenic mice. CR-1 can be detected in the plasma of MMTV-CR-1 mice during pregnancy or in transgenic mice with hyperplastic lesions or adenocarcinomas of the mammary gland. O.D., absorbance.

were done with the use of Statistical Package for Social Sciences software package, version 11.0 (SPSS, Chicago, IL). All statistical tests were two-sided, and data were considered statistically significant when $P < 0.05$.

Results

Human CR-1 sandwich ELISA. We developed an antibody sandwich ELISA to detect CR-1 in human plasma samples. Antibody sandwich ELISA has been shown to be more sensitive than ELISA, in which the antigen is directly bound to the plate (28, 29). An anti-CR-1 monoclonal antibody (A10.B2.18) was

used as the capture antibody and an anti-CR-1 rabbit polyclonal antibody was used as the detection antibody. A secondary goat anti-rabbit polyclonal antibody, labeled with horseradish peroxidase, was then used and absorbance was measured at 450 nm. We used purified human CR-1 recombinant protein to generate a calibration curve. CR-1 was diluted in 1% milk to a concentration ranging from 0.037 to 20 ng/mL. The assay showed a linear relationship within this range (Fig. 1A). We next assessed cross-reactivities of mouse monoclonal and rabbit polyclonal anti-CR-1 antibodies. Because the EGF-like domain of CR-1 shares amino acid sequence similarity with members of the EGF family of peptides (3), we examined potential cross-reactivity with EGF, amphiregulin, heparin binding-EGF, or heregulin β 1. All of the recombinant proteins produced readings comparable with the background signal, even at concentrations 1,000-fold higher than CR-1 (Fig. 1B). These data confirm that this sandwich ELISA measures CR-1 with high specificity because it efficiently discriminates CR-1 from other similar proteins.

Detection of human CR-1 in the plasma of MMTV-CR-1 transgenic mice. Because human CR-1 is overexpressed in the mammary gland of MMTV-CR-1 transgenic animals and because these animals, after several pregnancies, develop mammary hyperplasias and papillary adenocarcinomas, we evaluated whether soluble CR-1 could be detected in the plasma of MMTV-CR-1 transgenic animals (25). A total of four MMTV-CR-1 transgenic mice for each group were analyzed. We were not able to detect any CR-1 in the plasma of FVB/N control mice because the anti-CR-1 monoclonal antibody (A10.B2.18) used in the sandwich ELISA could only react with human CR-1 transgene protein and not with endogenous mouse Cr-1 protein (data not shown), as previously described (17). As shown in Fig. 1C, human CR-1 was detected in the plasma of MMTV-CR-1 mice during pregnancy (0.047 ± 0.0035 ng/mL; mean \pm SD) and in mice with hyperplastic lesions of the mammary gland (0.041 ± 0.0056 ng/mL) or with mammary adenocarcinomas (0.052 ± 0.0021 ng/mL). Because the MMTV promoter induces the constitutive expression of high levels of CR-1 in the mammary gland of MMTV-CR-1 transgenic mice, no statistically significant

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Table 1. Plasma samples derived from cancer patients and healthy volunteers

(A) Tumor stage of patients with colon and breast cancer

| Colon cancer (n = 33) | Breast cancer (n = 54) |
|-----------------------|------------------------|
| Stage I (9) | Stage I (13) |
| Stage IIA (3) | Stage IIA (21) |
| Stage IIIB (10) | Stage IIB (17) |
| Stage IIIC (5) | Stage IV (3) |
| Stage IV (6) | |

(B) Concentration of plasma CR-1 in healthy volunteers and patients with cancer

| Patients | No. of plasma samples | CR-1 concentration (ng/mL) | | | P* |
|----------------|-----------------------|----------------------------|-------------|--------|---------|
| | | Range | Mean (SD) | Median | |
| Healthy donors | 21 | 0.07-0.9 | 0.32 (0.19) | 0.27 | |
| Colon cancer | 33 | 0.86-19.18 | 4.68 (3.5) | 4.98 | <0.0001 |
| Breast cancer | 54 | 1.19-6.72 | 2.97 (1.48) | 2.02 | <0.0003 |

NOTE: Stage grouping according to the American Joint Committee on Cancer.

*Mann-Whitney U test was used to compare values in patients with cancer to values in the control group.

differences in CR-1 levels were observed among these three groups of animals. Therefore, we conclude from these results that a sandwich ELISA for CR-1 can specifically detect human CR-1 in biological fluids, such as plasma.

Identification of CR-1 in plasma of patients with cancer. CR-1 levels were analyzed in 87 plasma samples derived from cancer patients with either colon carcinoma ($n = 33$) or breast carcinoma ($n = 54$) at different clinical stages (Table 1A). We also examined 21 plasma samples from healthy subjects. As shown in Table 1B and Fig. 2A, very low levels of CR-1 (mean \pm SD) were detected in the plasma of healthy volunteers (0.32 ± 0.19 ng/mL). CR-1 levels in the plasma of patients with colon and breast carcinomas were significantly higher when compared with the control group ($P < 0.001$). The highest plasma levels of CR-1 were detected in colorectal cancer (4.68 ± 3.5 ng/mL; Table 1B). As a cutoff level, we chose the average of CR-1 values in the control group +2 SD (0.7 ng/mL). At this cutoff level, the test was positive in the plasma of all patients with cancer

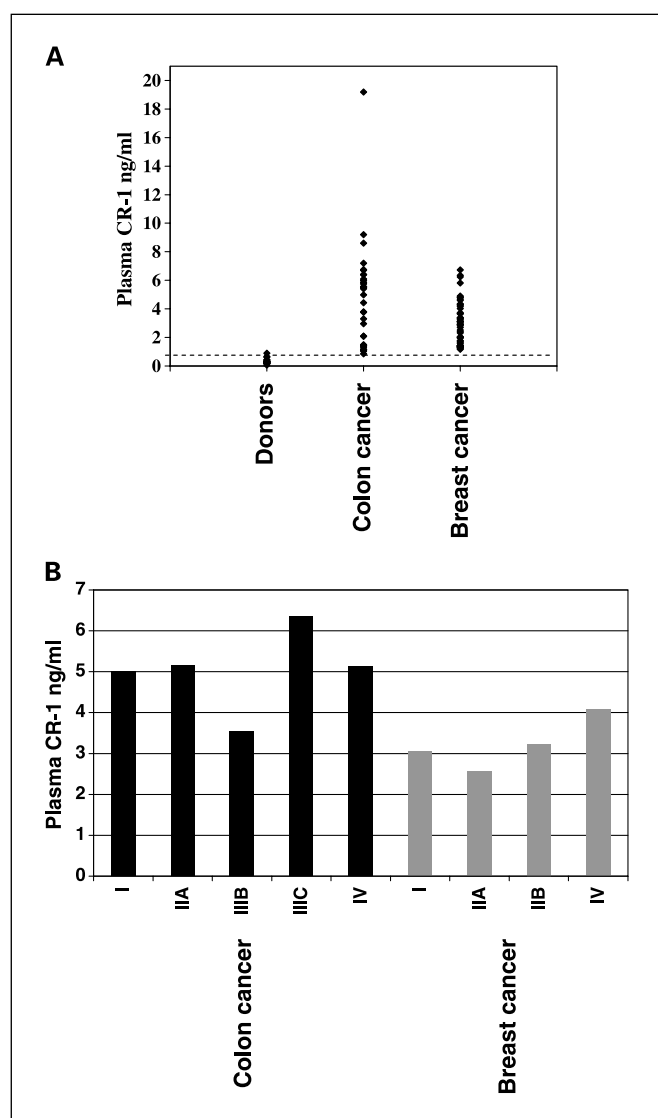


Fig. 2. A, plasma CR-1 levels (ng/mL) in patients with colon and breast cancer compared with normal donors. The cutoff value (---) corresponds with the mean value calculated in the control group +2 SD (0.7 ng/mL). B, concentration of plasma CR-1 in patients with colon and breast cancer at different tumor stages.

(100% sensitivity), and in 1 of 21 healthy donors (95% specificity). The accuracy of the test was 99%. No statistically significant differences for CR-1 plasma levels were found among patients with different stages of disease (Fig. 2B). Similarly, using two different univariate analysis tests, no statistically significant correlations were observed between CR-1 concentration in the plasma and various clinicopathologic variables in patients with breast and colon cancer, including tumor size, lymph node involvement, proliferative index as assessed by Ki-67 staining, estrogen and progesterone receptor status, or erbB-2 status where appropriate (data not shown).

Detection of CR-1 in the plasma of patients with benign breast lesions. We next assessed the levels of CR-1 in the plasma of 21 patients with benign breast lesions. These patients had clinical and radiological findings that were highly suggestive for breast carcinoma. Cytologic examination of fine-needle aspirate biopsy was not informative, or revealed the presence of atypical cells suggestive of hyperplasia. Therefore, these patients underwent surgical excision of the breast lesions. The plasma levels of CR-1 in patients carrying benign breast lesions were 1.7 ± 0.99 ng/mL (Table 2). The difference between the mean plasma CR-1 levels detected in patients with benign breast lesions and mean plasma CR-1 levels detected in patients with breast carcinoma (2.97 ± 1.48 ng/mL) was statistically significant ($P < 0.001$; Fig. 3). Within the group of patients with benign breast lesions, a trend towards higher levels of CR-1 were found in a subset of these patients ($n = 7$) with additional histologic signs of sclerosis, although this difference was not statistically significant (Table 2). Interestingly, high levels of plasma CR-1 were also found in a small group of patients ($n = 6$) with fibroadenoma, a pathologic breast benign lesion that is characterized by the presence of epithelial hyperplasia and fibrosis (Table 2). The difference in the mean CR-1 plasma levels between the fibroadenoma group and the nonsclerotic lesions is statistically significant ($P < 0.05$).

When a level of 2 ng/mL was arbitrarily chosen to separate benign breast lesions from breast carcinomas, the test was positive in 34 of the 51 patients with stage I or stage II breast cancer (67%), and in only 5 of 21 patients with benign breast lesions (24%; $P < 0.001$). Interestingly, four out of five benign breast lesions that were positive for CR-1, as detected by sandwich ELISA, were also characterized by the presence of sclerosis.

Immunohistochemical localization of CR-1 in tumor tissues from cancer patients. We next evaluated whether soluble CR-1, which was detected in the plasma of patients with cancer, originated from the primary tumor. Using immunohistochemistry, we analyzed CR-1 expression in colon and breast tumor tissue sections derived from patients that had high plasma levels of soluble circulating CR-1 protein. Specific staining for CR-1 was detected in all the colon cancer ($n = 7$) and breast cancer ($n = 15$) tissue sections that were examined (Fig. 4A). Weak to negative staining for CR-1 was detected in normal breast or colon tissue adjacent to the malignant lesions (data not shown). However, the levels of CR-1 in the plasma of patients with colorectal and breast cancer were not found to be associated with the degree of positivity for CR-1 in tumor sections (data not shown).

Detection of CR-1 mRNA in normal and tumor breast tissues. Finally, we assessed the levels of expression of CR-1 mRNA in another set of normal and malignant human breast

Table 2. Concentration of plasma CR-1 in patients with benign breast lesions

| Benign breast lesions | No. of plasma samples | CR-1 concentration (ng/mL) | | |
|-----------------------|-----------------------|----------------------------|-------------|--------|
| | | Range | Mean (SD) | Median |
| Total | 21 | 0.14-3.76 | 1.7 (0.99) | 1.51 |
| Without sclerosis | 14 | 0.14-3.76 | 1.41 (0.86) | 1.38 |
| With sclerosis | 7 | 0.9-3.46 | 2.28 (1.03) | 2.75 |
| Fibroadenoma | 6 | 0.96-5.5 | 2.65 (1.6) | 2.32 |

tissues obtained by the Fox Chase Cancer Center by using real-time RT-PCR. Total RNA was obtained from normal and breast cancer tissues. As shown in Fig. 4B, CR-1 mRNA could be detected in normal breast tissue samples. More importantly, a large increase in the levels of CR-1 mRNA expression was observed in six human primary breast carcinomas as compared with normal breast tissue. These data are in agreement with the results from the plasma and immunohistochemical analysis for CR-1 expression in patients with breast cancer.

Discussion

Despite the significant improvements in cancer therapy which have been achieved in the last 20 years, the prognosis for patients with cancer is mainly affected by the stage of the disease at diagnosis. The majority of human cancers are curable if diagnosed at an early stage, whereas death is common when the disease is discovered at a late stage. Therefore, the discovery of biomarkers for early diagnosis represents a major effort of cancer research.

Different biological markers have been proposed for the early detection of cancer. For example, early diagnosis of colorectal neoplasia can be accomplished by detection in the feces of mutations in *ras* or *p53*, or of changes in microsatellites of DNA (30). However, these assays are costly, and they can only be done in specialized molecular biology laboratories. Urokinase plasminogen activator and its receptor in nipple aspirate fluid samples are independent predictors of the presence of breast cancer (31). The feasibility of this procedure is significantly affected by the availability to obtain nipple aspirates, and by compliance of the patients with this approach. Serum markers for early diagnosis of cancer offer several advantages including the low cost and the possibility of detection with rapid and simple tests. However, the levels of a serum marker generally reflect tumor burden, and for this reason, they are not sensitive enough to be used for screening and early diagnosis of cancer (32). Growth factors, including transforming growth factor α and insulin-like growth factors, have also been proposed as potential tumor markers (33). Although epidemiologic evidence suggests a role for insulin-like growth factors and their binding proteins in the pathogenesis of different cancers, the value of serum insulin-like growth factors and insulin-like growth factor binding proteins for early diagnosis has not yet been established (34). In addition, some studies have also suggested that transforming growth factor α serum levels are elevated in patients with cancer compared with healthy controls; however, the diagnostic and/or prognostic value of serum transforming growth factor α in patients with carcinoma has not yet been proven (33, 35).

This article provides, for the first time, evidence that CR-1 might represent a more reliable marker for the detection of

cancer. In fact, a significant increase in the plasma levels of CR-1 was observed in patients affected by two of the most frequent types of human carcinomas, breast, and colon, as compared with healthy volunteers. The sensitivity of the ELISA assay to detect patients with cancer was 100% with a specificity of 95%. Interestingly, higher CR-1 plasma levels were found in patients with breast cancer at an early stage of disease as compared with levels detected in patients with benign breast lesions and in healthy volunteers, suggesting that CR-1 might indeed be a useful marker in this setting. No correlation was found between tumor size and plasma levels of CR-1 in patients with colon and breast cancer. However, the absolute number of patients with advanced disease in our cohort was low compared with patients with early stage disease, and this might have affected our analysis. Furthermore, CR-1 exists in two biologically active forms as a cell membrane-associated, glycosylphosphatidylinositol-linked protein and as a soluble protein (7-9). No information is available on the mechanism(s) that regulate the processing of these two forms in human carcinomas as influenced by the expression of glycosylphosphatidylinositol-specific phospholipases by carcinoma cells. Finally, the amount of plasma CR-1 might be affected by the extent of necrosis within the tumor mass. In fact, no correlation was found between the intensity of staining in the primary tumor and the levels of CR-1 in the plasma of patients with cancer in whom this was assessed.

Our findings are in agreement with previous studies demonstrating that CR-1 is usually expressed in normal tissues at low levels, and that an increase in CR-1 expression is an early event in carcinogenesis (1, 4). In fact, no expression of CR-1 has been previously reported to occur in normal breast and colon

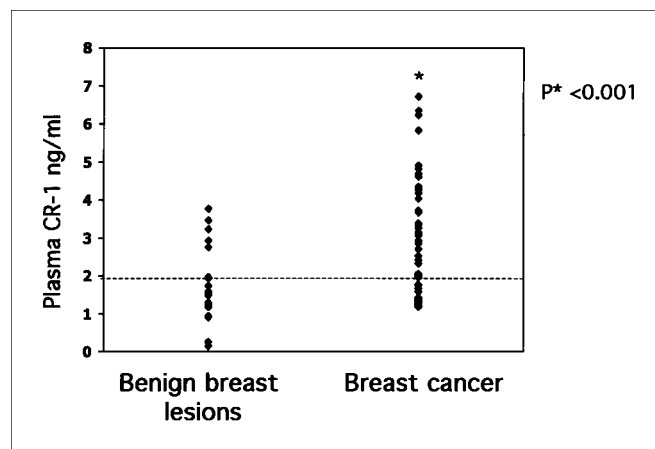
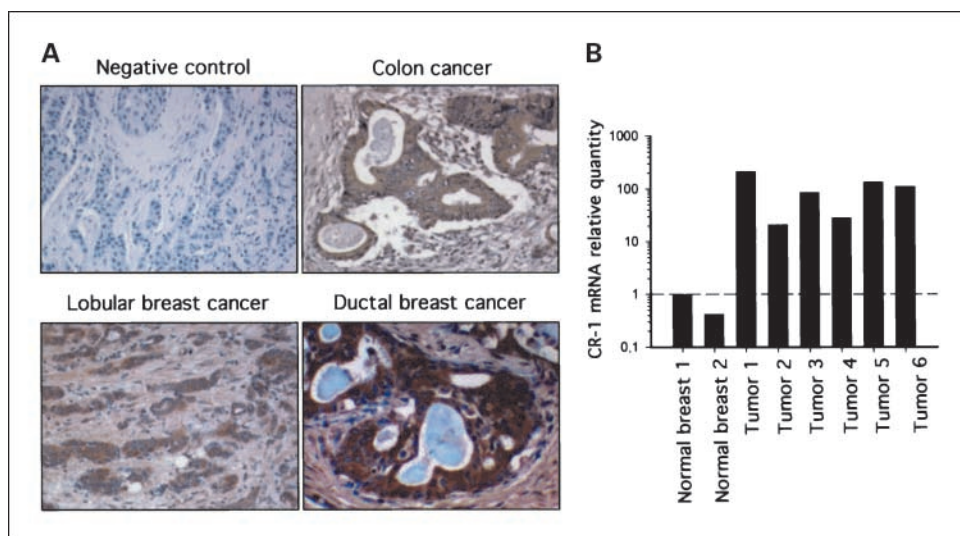


Fig. 3. Concentration of plasma CR-1 in women with benign breast lesions and in patients with breast cancer. The cutoff value (- - -) of 2 ng/mL was chosen to separate benign breast lesions from breast carcinomas.

Fig. 4. *A*, immunohistochemistry for CR-1 in matching tissue samples from patients with colon and breast carcinoma. In the negative control sample (breast cancer tissue) the slide was incubated with secondary antibody only. *B*, real-time RT-PCR in two human normal breast samples and in six human breast tumor samples. Relative quantification of CR-1 mRNA was done by using the $\Delta\Delta CT$ method, as explained in Materials and Methods.



tissue. In this regard, we found that CR-1 mRNA is expressed at low levels in normal human breast as compared with the levels of CR-1 mRNA in breast tumors. In contrast, a high frequency of CR-1 expression has been observed in colon tissue surrounding primary tumors and in the colon mucosa of individuals at high-risk, in whom CR-1 expression is associated with increased proliferation (36). The expression of CR-1 has also been observed in colon cancer premalignant lesions such as colon adenomas and in gastric metaplasia, suggesting that the expression of CR-1 may be a common event in human tumorigenesis (37, 38). In agreement with these findings, moderate levels of plasma CR-1 were found in women with non-cancer lesions of the breast including hyperplasia or atypical hyperplasia. In this subgroup of patients at high-risk, with clinical and radiological findings suggestive of breast carcinoma, preoperative levels of CR-1 might be a useful tool to help orient diagnosis and therapeutic intervention.

Within the nonmalignant breast lesions, the levels of plasma CR-1 were higher in fibroadenomas and in lesions characterized by the presence of sclerosis as compared with lesions

without sclerosis. Recent evidence from studies of fibrotic disorders, including renal, liver, and lung fibrosis, supports the view that a substantial number of fibroblasts which are found in these conditions are derived from epithelial cells through a phenomenon termed epithelial-to-mesenchymal transition (39–41). Interestingly, our group has previously shown that overexpression of CR-1 in mouse and human mammary epithelial cells leads to morphologic and functional modifications that are typical of epithelial-to-mesenchymal transition (19, 22). Therefore, we hypothesize that in nonmalignant breast lesions with sclerosis, CR-1 might have a role in inducing epithelial-to-mesenchymal transition in mammary epithelial cells (19). The implications of this phenomenon in breast tumorigenesis warrant further exploration.

In conclusion, for the first time, this study provides evidence that plasma levels of CR-1 are elevated in patients with colon and breast carcinomas. The observation that CR-1 levels are elevated in patients with early breast cancer suggests that this marker might be useful for the early detection of cancer. Further prospective studies are needed to substantiate this hypothesis.

References

- Bianco C, Strizzi L, Normanno N, Khan N, Salomon DS. Cripto-1: an oncofetal gene with many faces. *Curr Top Dev Biol* 2005;67:85–133.
- Salomon DS, Bianco C, Ebert AD, et al. The EGF-CFC family: novel epidermal growth factor-related proteins in development and cancer. *Endocr Relat Cancer* 2000;7:199–226.
- Bianco C, Normanno N, Salomon DS, Ciardiello F. Role of the cripto (EGF-CFC) family in embryogenesis and cancer. *Growth Factors* 2004;22:133–9.
- Strizzi L, Bianco C, Normanno N, Salomon D. Cripto-1: a multifunctional modulator during embryogenesis and oncogenesis. *Oncogene* 2005;24:5731–41.
- Kannan S, De Santis M, Lohmeyer M, et al. Cripto enhances the tyrosine phosphorylation of Shc and activates mitogen-activated protein kinase (MAPK) in mammary epithelial cells. *J Biol Chem* 1997;272:3330–5.
- Bianco C, Kannan S, De Santis M, et al. Cripto-1 indirectly stimulates the tyrosine phosphorylation of erb B-4 through a novel receptor. *J Biol Chem* 1999;274:8624–9.
- Minchiotti G, Parisi S, Liguori G, et al. Membrane-anchorage of Cripto protein by glycosylphosphatidylinositol and its distribution during early mouse development. *Mech Dev* 2000;90:133–42.
- Brandt R, Normanno N, Gullick WJ, et al. Identification and biological characterization of an epidermal growth factor-related protein: cripto-1. *J Biol Chem* 1994;269:17320–8.
- Normanno N, De Luca A, Bianco C, et al. Cripto-1 overexpression leads to enhanced invasiveness and resistance to anoikis in human MCF-7 breast cancer cells. *J Cell Physiol* 2004;198:31–9.
- Calhoun JD, Rao RR, Warrenfeltz S, et al. Transcriptional profiling of initial differentiation events in human embryonic stem cells. *Biochem Biophys Res Commun* 2004;323:453–64.
- Noaksson K, Zoric N, Zeng X, et al. Monitoring differentiation of human embryonic stem cells using real-time PCR. *Stem Cells* 2005;23:1460–7.
- Minchiotti G. Nodal-dependant Cripto signaling in ES cells: from stem cells to tumor biology. *Oncogene* 2005;24:5668–75.
- Normanno N, Bianco C, De Luca A, Salomon DS. The role of EGF-related peptides in tumor growth. *Front Biosci* 2001;6:D685–7.
- Normanno N, De Luca A, Maiello MR, et al. CRIPTO-1: a novel target for therapeutic intervention in human carcinoma. *Int J Oncol* 2004;25:1013–20.
- Normanno N, Bianco C, De Luca A, Maiello MR, Salomon DS. Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment. *Endocr Relat Cancer* 2003;10:1–21.
- De Luca A, Arra C, D'Antonio A, et al. Simultaneous blockage of different EGF-like growth factors results in efficient growth inhibition of human colon carcinoma xenografts. *Oncogene* 2000;19:5863–71.
- Adkins HB, Bianco C, Schiffer SG, et al. Antibody blockade of the Cripto CFC domain suppresses tumor cell growth *in vivo*. *J Clin Invest* 2003;112:575–87.
- Xing PX, Hu XF, Pietersz GA, Hosick HL, McKenzie IF. Cripto: a novel target for antibody-based cancer immunotherapy. *Cancer Res* 2004;64:4018–23.
- Strizzi L, Bianco C, Normanno N, et al. Epithelial

- mesenchymal transition is a characteristic of hyperplasias and tumors in mammary gland from MMTV-Cripto-1 transgenic mice. *J Cell Physiol* 2004;201:266–76.
20. Ciardiello F, Dono R, Kim N, Persico MG, Salomon DS. Expression of cripto, a novel gene of the epidermal growth factor gene family, leads to *in vitro* transformation of a normal mouse mammary epithelial cell line. *Cancer Res* 1991;51:1051–4.
 21. Ebert AD, Wechselberger C, Nees M, et al. Cripto-1-induced increase in vimentin expression is associated with enhanced migration of human Caski cervical carcinoma cells. *Exp Cell Res* 2000;257:223–9.
 22. Wechselberger C, Ebert AD, Bianco C, et al. Cripto-1 enhances migration and branching morphogenesis of mouse mammary epithelial cells. *Exp Cell Res* 2001;266:95–105.
 23. Bianco C, Strizzi L, Ebert A, et al. Role of human cripto-1 in tumor angiogenesis. *J Natl Cancer Inst* 2005;97:132–41.
 24. Sun Y, Strizzi L, Raafat A, et al. Overexpression of human Cripto-1 in transgenic mice delays mammary gland development and differentiation and induces mammary tumorigenesis. *Am J Pathol* 2005;167:585–97.
 25. Wechselberger C, Strizzi L, Kenney N, et al. Human Cripto-1 overexpression in the mouse mammary gland results in the development of hyperplasia and adenocarcinoma. *Oncogene* 2005;24:4094–105.
 26. Khan WN, Hammarstrom S. Biosynthesis of carcinoembryonic antigen (CEA) gene family members expressed in human tumor cell lines: evidence for cleavage of the glycosyl phosphatidyl inositol (GPI) anchor by GPI-PLC and GPI-PLD. *Biochem Int* 1991;25:723–31.
 27. Russo J, Balogh GA, Chen J, et al. The concept of stem cell in the mammary gland and its implication in morphogenesis, cancer and prevention. *Front Biosci* 2006;11:151–72.
 28. Hippo Y, Watanabe K, Watanabe A, et al. Identification of soluble NH₂-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. *Cancer Res* 2004;64:2418–23.
 29. Borgono CA, Grass L, Soosaipillai A, et al. Human kallikrein 14. A new potential biomarker for ovarian and breast cancer. *Cancer Res* 2003;63:9032–41.
 30. Srivastava S, Verma M, Henson DE. Biomarkers for early detection of colon cancer. *Clin Cancer Res* 2001;7:1118–26.
 31. Ross JS, Linette GP, Stec J, et al. Breast cancer biomarkers and molecular medicine: part II. *Expert Rev Mol Diagn* 2004;4:169–88.
 32. Seregini E, Coli A, Mazzucca N. Circulating tumour markers in breast cancer. *Eur J Nucl Med Mol Imaging* 2004;31 Suppl 1:S15–22.
 33. Chakrabarty S, Huang S, Moskal TL, Fritsche HA, Jr. Elevated serum levels of transforming growth factor- α in breast cancer patients. *Cancer Lett* 1994;79:157–60.
 34. Shi R, Yu H, McLarty J, Glass J. IGF-I and breast cancer: a meta-analysis. *Int J Cancer* 2004;111:418–23.
 35. Shim KS, Kim KH, Park BW, et al. Increased serum levels of transforming growth factor- α in patients with colorectal cancer. *Dis Colon Rectum* 1998;41:219–24.
 36. De Angelis E, Grassi M, Gullick WJ, et al. Expression of cripto and amphiregulin in colon mucosa from high risk colon cancer families. *Int J Oncol* 1999;14:437–40.
 37. Saeki T, Stromberg K, Qi CF, et al. Differential immunohistochemical detection of amphiregulin and cripto in human normal colon and colorectal tumors. *Cancer Res* 1992;52:3467–73.
 38. Tahara E, Kuniyasu H, Yasui W, Yokozaki H. Gene alterations in intestinal metaplasia and gastric cancer. *Eur J Gastroenterol Hepatol* 1994;6 Suppl 1:S97–102.
 39. Zeisberg M, Kalluri R. The role of epithelial-to-mesenchymal transition in renal fibrosis. *J Mol Med* 2004;82:175–81.
 40. Willis BC, Liebler JM, Luby-Phelps K, et al. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor- β 1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol* 2005;166:1321–32.
 41. Liu Y. Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. *J Am Soc Nephrol* 2004;15:1–12.