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June 28, 1996

Philip J. Migliore, M.D.
Chairrnan and Research Director
The Moran Foundation
Department of Pathology
Baylor College of Medicine
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Dear Dr. Migliore:

I am writing this letter to report to you, and to the Scientific Advisory Committee of the Moran Foundation, on the status of my projects supported by The Foundation.

I- "Specific Diagnosis of Celiac Sprue by Immunohistochemistry and/or Immunofluorescence" (4-93-0068):

This project is **inactive**: Unfortunately, I could not get clinical samples for a prospective study, because some clinical colleagues who promised to collaborate have dragged their feet for too long, and non of the promises materialized.

II- " Biopsy-based prediction of stage and prognosis in colorectal cancer":

This project has resulted in the following publication:

Younes M, Fernandez L, Lechago J: Transforming growth factor alpha (TGF-a) expression in biopsies of colorectal carcinoma is a significant prognostic indicator. Anticancer Res 1996 (in press).

Support of the Moran Foundation is acknowledged in that paper.

One or two more publications may result from additional work in progress related to this project. In the case of publication, the Foundation will be, of course, acknowledged.

Thank you for your support.
Sincerely,

Mamoun Younes

Mamoun Younes, M.D.

TRANSFORMING GROWTH FACTOR ALPHA (TGF-α) EXPRESSION IN BIOPSIES OF COLORECTAL CARCINOMA IS A SIGNIFICANT PROGNOSTIC INDICATOR

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Key words: preoperative biopsy, survival, colon cancer, immunohistochemistry, TGF-α

Running title: TGF-a in colon cancer biopsies.

CLINICAL STUDY

Supported in part by the Moran Foundation

Presented in part at the 86 th annual meeting of the American Association for Cancer Research in Toronto, Canada, March 1995.

DATE OF SUBMISSION: FEBRUARY 7, 1996

ABSTRACT:

Background: Predicting the outcome of patients with colorectal adenocarcinoma (CRCA) prior to surgery would be valuable in selecting high-risk individuals who may benefit from pre-operative adjuvant therapy. The aim of this study is to determine whether the expression of TGF-α in preoperative biopsies of patients with CRCA constitutes a significant prognostic indicator. **Methods:** We studied the expression of TGF-a in preoperative biopsies of 106 patients with CRCA, who had at least 5 years follow-up, using an anti-TGF-a monoclonal antibody and utilizing the ABC immunoperoxidase technique. For survival analysis, we used the actuarial survival method, and the Log Rank test for statistical significance. **Results:** CRCAs with low TGF-α expression (less than 25% of the tumor cells immunoreactive for TGF-α) had a significantly poorer survival than those with high TGF-α expression (more than 25%). After excluding from analysis biopsies showing mucinous or poorly differentiated CRCA, known predictors of poor prognosis, the results remained significant ($p=0.0289$). **Conclusion:** It is concluded, therefore, that low or absent expression of TGF-α in pre-operative biopsies of patients with CRCA, as detected by immunohistochemistry, is a significant predictor of an unfavorable outcome.

INTRODUCTION

Colorectal adenocarcinoma is the second most common malignancy in females and third in males. In 1989, 57,382 Americans died of this cancer, and it has been estimated that, in 1993, 152,000 new cases of colorectal cancer would be diagnosed and 57,000 deaths would be caused by it (1). Despite significant advances in surgery, as well as in radiation and chemotherapy, the mortality of colorectal carcinoma has remained steady over the past 40 years (2), although recent reports show a decrease in the incidence and mortality of rectal carcinoma attributed to surveillance and early detection (3). In order to make an impact on the mortality from this cancer, it has been recommended that research efforts include the development of indicators of the biological activity of these tumors in order to improve the pre-and post-operative staging (4). Such improved staging would result in better and more accurate selection of patients for the different available therapeutic modalities, based on their risk to benefit ratio.

Recently, more attention is being paid to neo-adjuvant (induction) therapy, which has shown promising results in advanced breast (5,6) and bladder (7) cancer. In contrast, clinical trials on colorectal carcinoma patients have yielded conflicting results, perhaps as a result of the inability to identify pre-operatively those patients with advanced tumors who are likely to benefit from such therapy (8). Similarly, failure to accurately predict the extent of some rectal cancers preoperatively has been blamed for the failures occasionally seen in conservative surgery such as transanal resection (9,10). Having a reliable predictor of outcome of patients with colorectal carcinoma should be

of great help to treating physicians in choosing the best of available therapeutic options based on the pre-operative biopsy. This will be also helpful in selecting the most appropriate course of action for elderly patients with a biopsy diagnosis of colon cancer, in whom surgery caries a significant morbidity and mortality (11).

Transforming growth factor-Alpha (TGF-α) is a polypeptide that produces a mitogenic effect through interaction with the epidermal growth factor receptor (12), and is belived to promote carcinogenesis in the liver (13-16). TGF-α is secreted by many colon cancer cell lines (17-20), and was found to promote the growth of colon cancer cells in vitro (21-23), acting as an autocrine growth factor. The aim of this work is to determine to what degree TGF-α expression in preoperative biopsies of colorectal carcinomas correlates with the aggressiveness of these cancers, and thus can be used as a predictor of patient outcome following surgery.

MATERIALS AND METHODS

PATIENTS: A total of 105 patients with colorectal adenocarcinoma, diagnosed and treated at The Methodist Hospital (TMH) in Houston, TX during the years1984-1989, were entered in the study. All of these patients were treated with surgery alone. The mean follow-up was 80 months, and the median 69 months. Follow up information was obtained from the Cancer Registry at TMH.

HISTOLOGY: Hematoxylin and eosin-stained sections of preoperative biopsies were reviewed and evaluated for mucin production and histologic differentiation.

IMMUNOHISTOCHEMISTRY: Sections of formalin-fixed and paraffin-embedded biopsies were cut and mounted on Fisher Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), and heated at 58°C for 4 hours. Sections were deparaffinized in xylene and rehydrated through decreasing concentrations of alcohol ending in PBS. Sections were then incubated with 0.05% saponin (Sigma Chemical Company, St Louis, MO) for 30 min at room temperature (RT), and washed with PBS X3. The sections were incubated with 2% normal horse serum in 1% BSA/PBS for 30 minutes at RT, washed in PBS, and incubated with anti TGF-α antibody (Ab-2, Oncogene Science, Uniondale, NY) diluted 1:50 in 0.1% BSA in PBS overnight at 4°C. The sections were washed in PBS, and the bound antibody was detected using Vectastain Elite ABC mouse kit (Vector Laboratories, Burlingame, CA), and DAB as the chromogen. Sections were counterstained with hematoxylin, dehydrated, and

coverslipped using Accumount (Fisher) as the mounting medium. Slides incubated with 0.1% BSA in PBS instead of the primary antibody were used as negative control.

EVALUATION OF THE IMMUNOSTAINING: The percent of cancer cells which stained for TGF-α was semiquantitatively scored as a) 0 (negative), b) <10%, c) 10-25%, d) 25-50%, e) 50-75%, or f) >75%. A cut-off value of 25% was reached by analyzing the data using all above scores as cut-off values. The cut-off value 25% produced the most significant result (least p value) by the log rank test.

SURVIVAL ANALYSIS: was performed by the actuarial survival method and the log rank test for statistical significance, using StatView statistical software for the Macintosh with Survival Tools, version 4.5.

RESULTS:

Positive TGF-α staining was present in the cytoplasm of the tumor cells. The number of positive cells varied, and so did the intensity of staining. In all cases, when an area of the tumor was positive, all cells in that area were positive. Normal epithelial cells, when present in the same biopsy, also showed positive cytoplasmic staining for TGF-α. Examples of a positive and a negative case are shown in figure 1.

When all carcinomas were considered in the analysis, cases with less than 25% of cancer cells positive for TGF-α were associated with worse outcome than those with >25% TGF-a-positive cells ($p= 0.0412$) (Figure 2). When poorly differentiated carcinomas and mucinous carcinomas were excluded from the analysis, the difference in survival remained significant ($p= 0.0289$) (Figure 3). No association was found between the percent of TGF-α positive cancer cells in the biopsy and the stage of the corresponding resected tumors.

DISCUSSION

Because of the reported effects of TGF-α as a promoter of growth of colon cancer cells in vitro (21-23), it appeared somewhat paradoxical to find that decreased TGF-α expression is actually associated with a poorer survival rate. However, Markowitz at al. showed that TGF-α and the epidermal growth factor receptor are co-expressed in normal colonic epithelium as well as colonic adenomas, and they concluded that TGF-α is an important physiologic stimulant of normal epithelial proliferation (24). Moreover, it has been recently shown that TGF-α can enhance the differentiation of a colon cancer cell line grown in 3-dimentional collagen gel (25), and that induction of terminal differentiation of human colon carcinoma cells is associated with a 20-fold induction of TGF-α (26). These findings seem to indicate that TGF-α is the physiologic growth and differentiation stimulator of the normal colon epithelium. Therefore, perhaps the loss TGF-α indicates loss of contro! over the physiologic growth and differentiation mechanism, and this is probably the reason why its absence is associated with a poor prognosis. This is similar to the poor outcome associated with the loss of estrogen receptors in breast cancer, while estrogen interaction with these receptors promotes growth of normal breast epithelial cells (physiologic growth promoter), as well as breast cancer cell lines in vitro.

Our results show that patients with colorectal carcinoma whose preoperative biopsy shows <25% of the cancer cells to be positive for TGF-α are likely to have a

significantly worse prognosis than those with a biopsy containing >25% TGF-α-positive cells (p=0.0289). We conclude that the use of molecular prognostic markers, such as immunostaining for TGF-α, may be very useful in predicting the outcome of patients with colorectal carcinoma pre-operatively and therefore selecting the appropriate therapy, and in planning further clinical trials with neo-adjuvant therapy.

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FIGURE LEGEND:

Figure 1. Immunohistochemical staining for TGF-α in biopsies of colorectal adenocarcinomas, showing typical cytoplasmic staining. A, positive biopsy. B, negative biopsy. Immunoperoxidase staining, counterstained with hematoxylin (X 200).

Figure 2. Overall survival for all patients with colorectal adenocarcinoma, according to the percentage of TGF-α-positive cancer cells in the pre-operative biopsies.

Figure 3. Overall survival for patients with colorectal adenocarcinoma, excluding those with biopsies showing mucinous or poorly differentiated carcinoma, according to the percentage of TGF-α-positive cancer cells in the pre-operative biopsies.

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ELSEVIER

The Prognostic Value of Estrogen Receptor Immunocytochemistry (ERICA) in Breast Cancer Does Not Depend on the Immunostaining Intensity

Mamoun Younes, MD, Rodolfo Laucirica, MD, Charles C. Miller, PhD, and Richard W. Brown, MD

KEY WORDS: Estrogen receptor, breast cancer, survival, prognosis, immunohistochemistry

Estrogen receptor status (ER) is traditionally determined by the immunocytochemical assay (ERICA) utilizing a formula, the HSCORE, based on the stain intensity and the percentage of ER-positive cells (%PC). The stain intensity is difficult to control and its determination is subjective. To determine whether %PC alone can be used instead of HSCORE in determining ER status in breast cancer, frozen samples from 65 breast cancers were assayed for ER by the dextran-coated charcoal assay (DCCA), HSCORE, and %PC. %PC and HSCORE correlated well. ER status determined by any of the three methods was a significant prognostic factor. When applied to paraffin sections of a different group of 91 cancers, ER %PC and DCCA did not give significant results. We conclude that (1) %PC may be used instead of HSCORE for determining ER in tissue sections and (2) inconsistencies of the prognostic significance of ER may be due to differences in populations studied.

Introduction

Estrogen receptor (ER) status has been found to be a significant predictor of survival (1–3) and of response to hormonal therapy (4,5) in women with breast cancer. Traditionally, ER has been determined biochemically using a radiolabeled ligand (6). Although the dextran-coated charcoal assay (DCCA) is a well-established quantitative and objective biochemical method for the determination of ER status, it has many disadvantages (6), including the minimum of 200 mg of tumoral tissue required for adequate determination of ER (6). Since the most notable increase in the incidence of breast cancer in recent years has been attributed to an increase in the incidence of small

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cancers (7), thereby limiting the amount of tissue available for DCCA, an ER immunocytochemical assay (ERICA) that can be applied to histologic sections has been established (8–16). Although most studies have focused on the correlation between ERICA and DCCA (8–15), few studies have addressed the value of ERICA as a prognostic indicator (16–19). Because the quantity of ER in breast cancer is important (4), semiquantitative manual and computer-assisted methods have been developed for the determination of ER status by ERICA (13–15), based on a formula that takes into account both the intensity of the immunostaining and the percentage of ER-positive cancer cells (%PC). Determination of immunostaining intensity is very subjective when performed manually and has significant variability whether determined manually or by image analysis. Sources of variability in the stain intensity include batch-to-batch variability, which depends on the technician, materials, and methods used, and variability in the thickness of the tissue sections. In an attempt to address this complicated problem, Battifora et al. have suggested embedding a control gel containing an ER-positive breast cancer cell line in the same tissue cassette with each breast cancer specimen to provide an internal standard (20). Although this technique may control for most of the variables related to the stain intensity, it is impractical for most laboratories. A few studies have shown that the percentage of ER-positive cancer cells (%PC) is a significant prognostic marker in breast cancer (17,18), indicating that the stain intensity may not be a crucial factor in determining the prognostic value of ER.

The aim of this study was to determine whether %PC can be used instead of the HSCORE to evaluate ER status in breast cancer, using prognostic value and DCCA as means of comparison.

Materials and Methods

Patients

Study on Frozen Tissues Sixty-five women with breast cancer with a mean age of 66 years (range 37–91 years) were entered in this study. The criteria for inclusion in this study were as follows: (a) Patients had invasive breast cancer that was being treated at Methodist Hospital in Houston, (b) had a biochemical assay for estrogen receptors done at the ER laboratory at Baylor College of Medicine, (c) had frozen tumor tissue available, and (d) agreed to a minimum follow-up of one year. Perioperative deaths, tumors of special types, and patients with more than one breast cancer (including bilateral) were excluded from the study. Tumor size was obtained from the pathology reports. Nuclear pleomorphism and the degree of differentiation were assessed by a single pathologist (R.L.) on hematoxylin and eosin (H&E)–stained tissue sections according to the Scarff–Bloom–Richardson criteria (21,22). Follow-up information was obtained from the Cancer Registry at Methodist Hospital.

Study on Formalin-Fixed and Paraffin-Embedded Tissues Ninety-one women with invasive breast cancer with a mean age of 62 years (range 28–90) were entered in the study, after applying the same inclusion and exclusion criteria detailed above, with the exception that available frozen tissue was not a requirement for inclusion in the study. Blocks of formalin-fixed and paraffin-embedded tissue of the resected cancers, and H&E-stained sections were retrieved. Size, nuclear pleomorphism, differentiation, and follow-up information were obtained as above.

Biochemical Determination of Estrogen Receptor Status by the Dextran-Coated Charcoal Assay (DCCA) We used the sucrose gradient centrifugation method (6,23)

on frozen breast cancer tissues that were snap-frozen within 30 min of removal. The ligand used was 16a[^{125}I]-iodo 3, 17β-estradiol (NEN DuPont, Wilmington, DE).

Preparation of Tissue sections The unused portion of the breast cancer tissue submitted for the DCCA was kept frozen in liquid nitrogen. The frozen tissue was embedded in OCT compound (Miles, Elkhart, IN) and snap-frozen in liquid nitrogen. Then 6-μm-thick cryostat sections were cut on Fisher Superfrost plus slides (Fisher Scientific, Pittsburgh, PA), immediately fixed in Zamboni's fixative for 10 min at room temperature (RT), then washed in 20% sucrose in PBS and frozen at −80°C until used for staining. Five-μm-thick sections of the formalin-fixed and paraffin-embedded tissues were cut on Fisher Superfrost Plus slides and heated at 58°C for 4 hr.

ER Immunocytochemical Staining (ERICA) Frozen Sections: All procedures were carried out at RT, and all washings were in PBS 5 min × 3. Sections were washed, incubated with 3% normal goat serum in PBS for 30 min, washed, and incubated with a 1:40 dilution of antiestrogen antibody in 0.1% BSA in PBS (NCL-ER-LH1, Novo Castra, obtained through Vector Laboratories, Burlingame, CA) or 1% BSA in PBS (negative control) for 60 min. The bound antibody was detected by standard streptavidin-biotin technique using a prediluted StrAviGen Multilink HRP kit (Bio Genex, San Ramon, CA) and DAB for color development. The sections were then counterstained with methyl green (Sigma Chemical Company, St. Louis, MO), dehydrated through increasing concentrations of alcohol, cleared in xylene, and mounted and coverslipped using Accu Mount medium (Baxter Scientific, McGaw Park, IL). Sections of recent cases known to be ER-positive by the DCCA were used as positive control.

Paraffin sections: Sections of formalin-fixed, paraffin-embedded tissue were deparaffinized in xylene and rehydrated through decreasing concentrations of alcohol ending in PBS. Sections were microwaved in 10 mM citrate buffer pH 6.0 for 5 min × 3, washed in PBS, blocked with 2% normal goat serum in 1% BSA in PBS for 30 min at room temperature (RT), washed in PBS, and incubated overnight at 4°C with a 1:40 dilution of antiestrogen antibody in 0.1 BSA in PBS (1D5, AMAC, Inc., Westbrook, ME). The sections were washed in PBS, and the bound antibody was detected using a prediluted StrAviGen Multilink HRP kit (BioGenex) and DAB for color development. The sections were then counterstained with methyl green (Sigma), dehydrated through increasing concentrations of alcohol, cleared in xylene, and mounted and coverslipped using Accu Mount medium. Sections of recent cases known to be ER-positive by the DCCA were used as positive control.

Evaluation of ERICA The immunocytochemically stained sections were evaluated by one pathologist (R.B.) without prior knowledge of patient survival or the results of the DCCA. Two separate parameters were evaluated for each tumor, the percentage of ER-positive cancer cells (%PC), and HSCORE (15). HSCORE = $\Sigma(i + 1) \times Pi$, where i is the staining intensity (0, 1+, 2+, 3+, 4+), and Pi is the percentage of cells in each staining intensity. Up to 612 cancer cells were evaluated in each case (depending on the size of the tumor in the section; the minimum was 120 cells). Only nuclear staining was regarded as a positive result. No attempt was made to control for the thickness of tissue sections.

Statistical Analysis We used the actuarial survival method and the logrank test for statistical significance. Cutoff values used in this study are those that produced the

lowest p value when the survival analysis and the logrank test were performed several times using multiple cutoff values. Regression analysis was used for the correlation between %PC and HSCORE, and Fisher's exact test was used to determine the significance of differences in size, nuclear pleomorphism, and differentiation. Statistical analysis was performed using StatView for the Macintosh with Survival Tools version 4.1.

Results

Frozen Tissues, All Tumors

Most tumors had either < 10% or > 80% of the cells positive for ER by ERICA (Fig. 1), and there was an excellent correlation between %PC and HSCORE ($R = 0.946$, $p < 0.0001$) (Fig. 2). Survival analysis showed that patients with ER > 7.5 fmol/mg protein, as determined by DCCA, had better survival than those with ER < 7.5 fmol/mg ($p = 0.0109$, Fig. 3A). When ER was determined by ERICA, patients with HSCORE > 85.5 had better survival than those with HSCORE < 85.5 ($p = 0.0341$, Fig. 3B), and patients with %PC > 82% had better survival than those with %PC < 82% ($p = 0.0275$, Fig. 3C).

Frozen Tissues, Node-Negative Tumors

The number of patients in this category was 31. Survival analysis, using the same cutoff values as above, showed that no deaths occurred among the patients with node-negative cancers who had ER values above the cutoff values, whereas all deaths were among the patients with ER values below the cutoff values, regardless of the method used for ER determination (Fig. 4A–C). The p value for any of these curves could not be determined by the rank test because the groups with the higher ER values as determined by any of the three methods contained no uncensored observations.

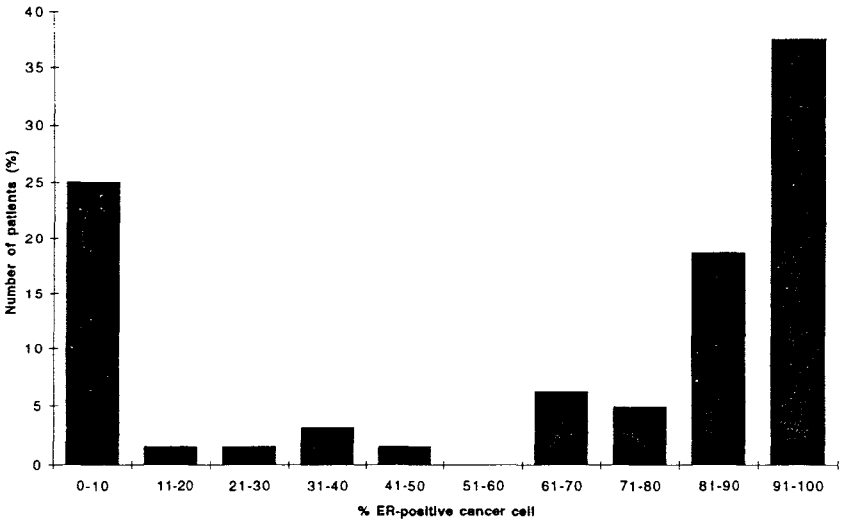


Figure 1. Percentage of ER-positive tumor cells (%PC) in 65 invasive breast carcinomas evaluated by immunoperoxidase staining on frozen sections. Note that 25% of the patients have tumors with %PC < 10% and 58% have %PC > 80%.

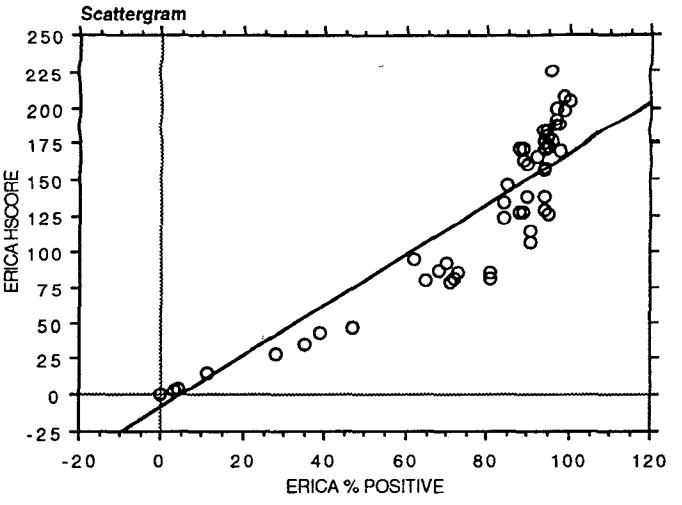


Figure 2. Correlation between ERICA %positive cells (%PC) and ERICA HSCORE in 65 patients with invasive breast cancer. $R = 0.946$, $p < 0.0001$.

Formalin-Fixed and Paraffin-Embedded Tissues, All Tumors

Most tumors had either < 10% or > 80% of the cells positive for ER by ERICA (Fig. 5). Survival analysis using the same cutoff values as for frozen tissues showed no significant difference in survival between patients with breast cancers having ER > 7.5 fmol/mg and those with < 7.5 fmol/mg ($p = 0.8193$, Fig. 6A). Likewise, there was no significant difference in survival between patients with cancers having ERICA %PC > 82% and those with %PC < 82% ($p = 0.6154$, Fig. 6B). Several additional cutoff values for %PC, including those most widely applied in previous studies (5% and 10%), were also tested and failed to produce statistical significance.

Formalin-Fixed and Paraffin-Embedded tissues, Node-Negative Tumors

The number of patients in this category was 51. No significant difference in survival was observed between patients with breast cancers having ER > 7.5 fmol/mg and those with < 7.5 fmol/mg ($p = 0.5097$, Fig. 7A). Similarly, there was no significant difference in survival between patients with cancers having ERICA %PC > 82% and those with %PC < 82% ($p = 0.7294$, Fig. 7B). When different cutoff values were tested, a statistically significant difference in survival was obtained with an ER-DCCA value of 25 fmol/mg ($p = 0.0414$). However, there was no statistically significant difference in survival for any %PC cutoff value tested. The lowest p value was obtained with a %PC cutoff value of 89.5% ($p = 0.1601$).

Size, Nuclear Grade, and Tubule Formation in Node-Negative Tumors

Tumor size was obtained on 25 frozen tumors and 45 paraffin-embedded tumors. Twenty of the 25 frozen tumors (80%) were ≥ 2.0 cm, whereas 20 of the 45 formalin/paraffin tumors (44%) were ≥ 2.0 cm ($p = 0.0054$). More frozen tumors showed moderate to high nuclear pleomorphism (grades 2 and 3) than formalin/paraffin tumors, but this

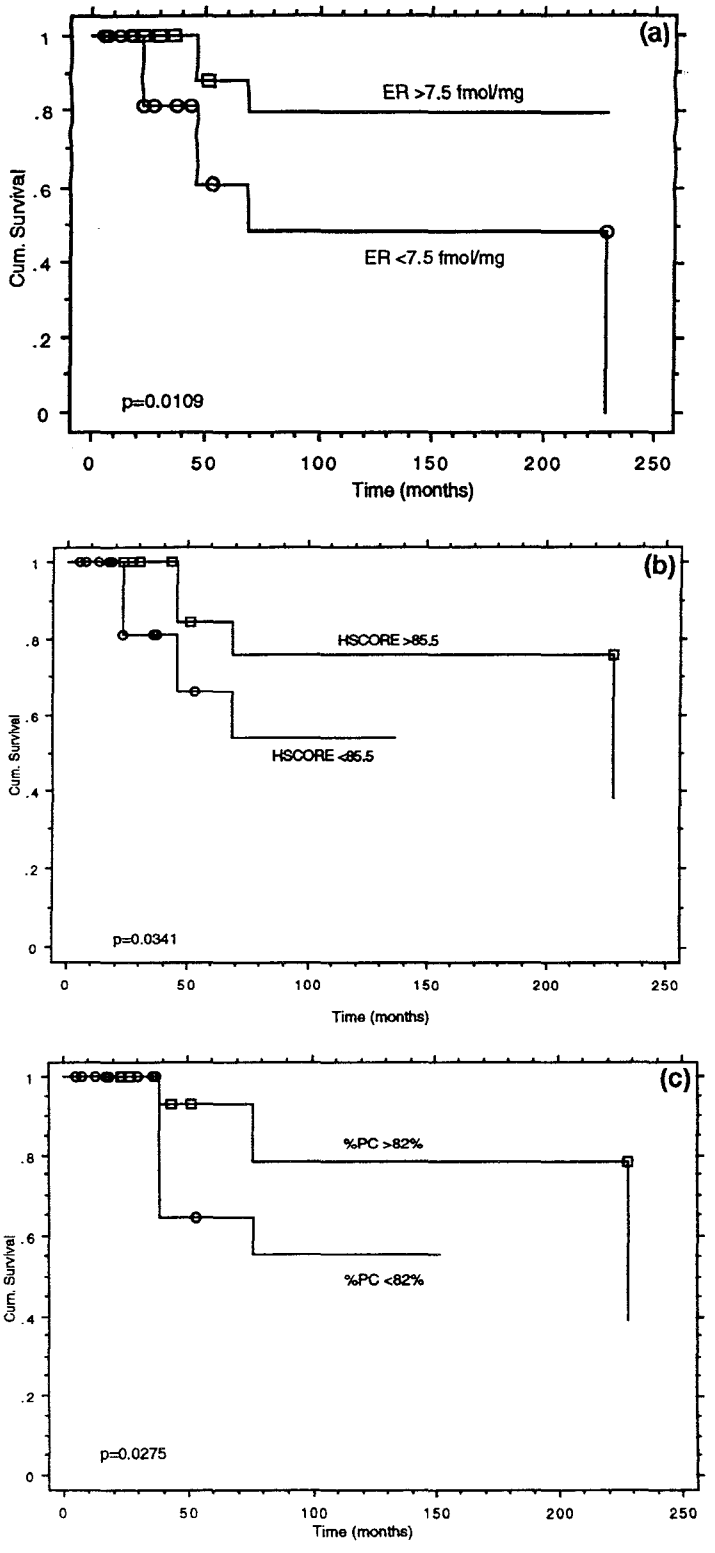
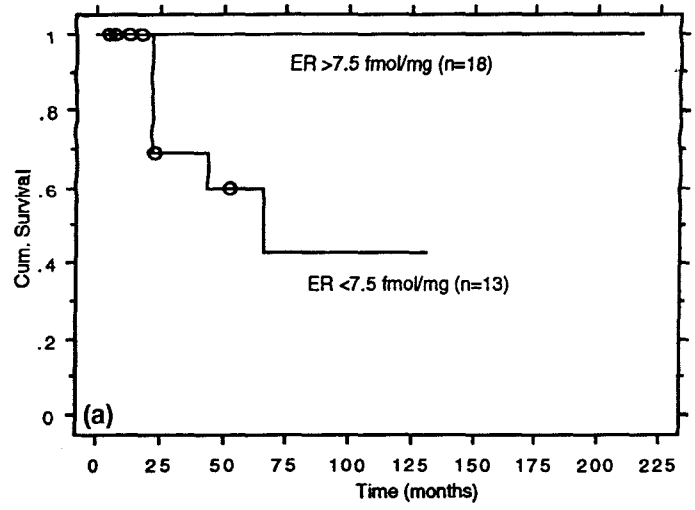
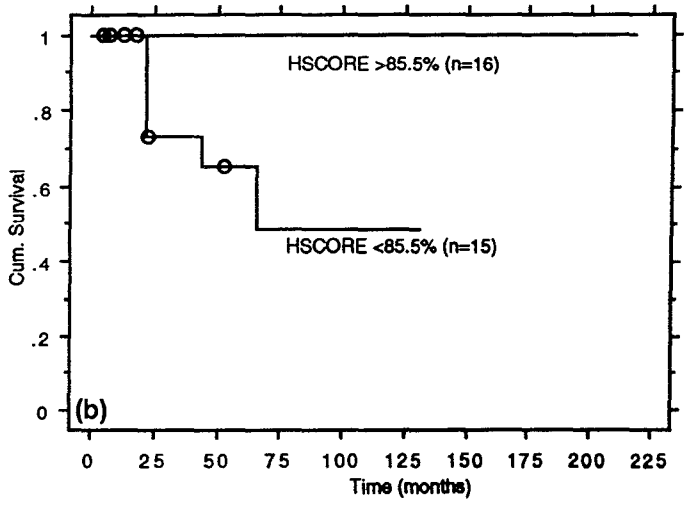


Figure 3. Overall survival of patients with invasive breast cancer according to ER status, as determined by the dextran-coated charcoal assay (DCCA) (A), and frozen section ERICA evaluated by HSCORE (B) or %PC (C).





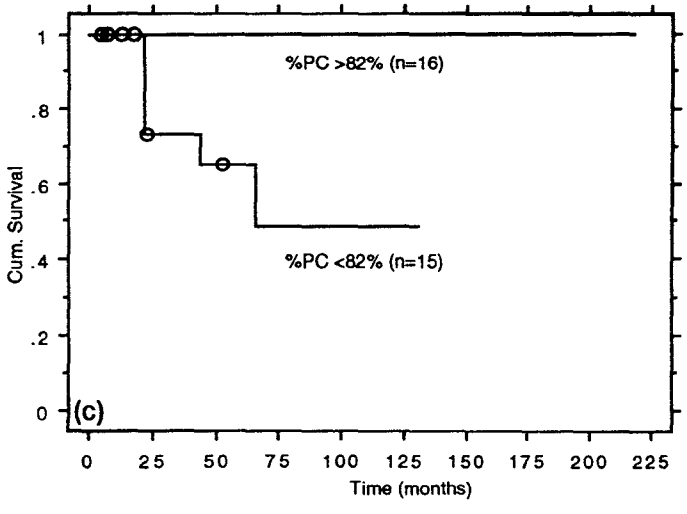


Figure 4. Overall survival of patients with node-negative invasive breast cancer according to ER status, as determined by the dextran-coated charcoal assay (DCCA) (A), and frozen section ERICA evaluated by HSCORE (B) or %PC (C).

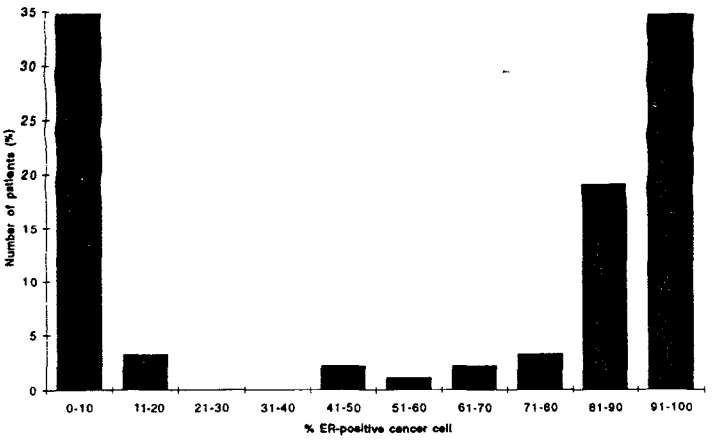


Figure 5. Percentage of ER-positive tumor cells (%PC) in 91 invasive breast carcinomas evaluated by immunoperoxidase staining on formalin/paraffin sections. Note that 35% of the patients have tumors with %PC < 10% and 54% have %PC > 80%.

difference was not significant (24 of 31, or 78%, vs. 35 of 51, or 69%, respectively; $p = 0.4544$). In addition, more tumors were moderately to poorly differentiated (tubule formation score 2 or 3) than formalin/paraffin tumors; however, the difference was not significant (28 of 31, or 90%, vs. 39 of 51, or 76%, respectively; $p = 0.147$).

Discussion

Breast cancer is the most common cancer and second most common cause of cancer deaths in women in the United States (24,25). It has been estimated that in 1993, 182,000 new cases of breast cancer will be diagnosed and 46,000 women will die of this disease (25). Decreasing the mortality from breast cancer will require a combination of prevention, early diagnosis, and therapy. For therapy to be appropriate, an analysis of the risk-to-benefit ratio should be done before therapeutic decisions are made. Risk assessment depends largely on the analysis of several prognostic markers of which ER status is one of the few established and clinically used markers (26). Most clinical trials and survival analyses using ER as a prognostic marker or as predictor of response to hormonal therapy have largely depended on DCCA. This assay has many problems (6), the most important of which are the contradicting reports about its utility as a prognostic indicator (17,18,25) and the required sample size of more than 200 mg of tumoral tissue (6), which renders a large proportion of breast cancers in today's practice unassayable for ER by this method.

The contradicting reports on the prognostic significance of ER determined by DCCA may be largely attributed to sampling, as well as technical variability between different laboratories. The sampling problem is unavoidable, mainly because the biochemical assay is based on measuring the cytosolic ER in fmol/mg protein in tissue homogenate. The ER value, measured as fmol/mg protein, may be influenced by contaminating nonmalignant epithelial cells that may express ER (28,29). ER in these normal cells may vary depending on the phase of the menstrual cycle and use of exogenous hormones

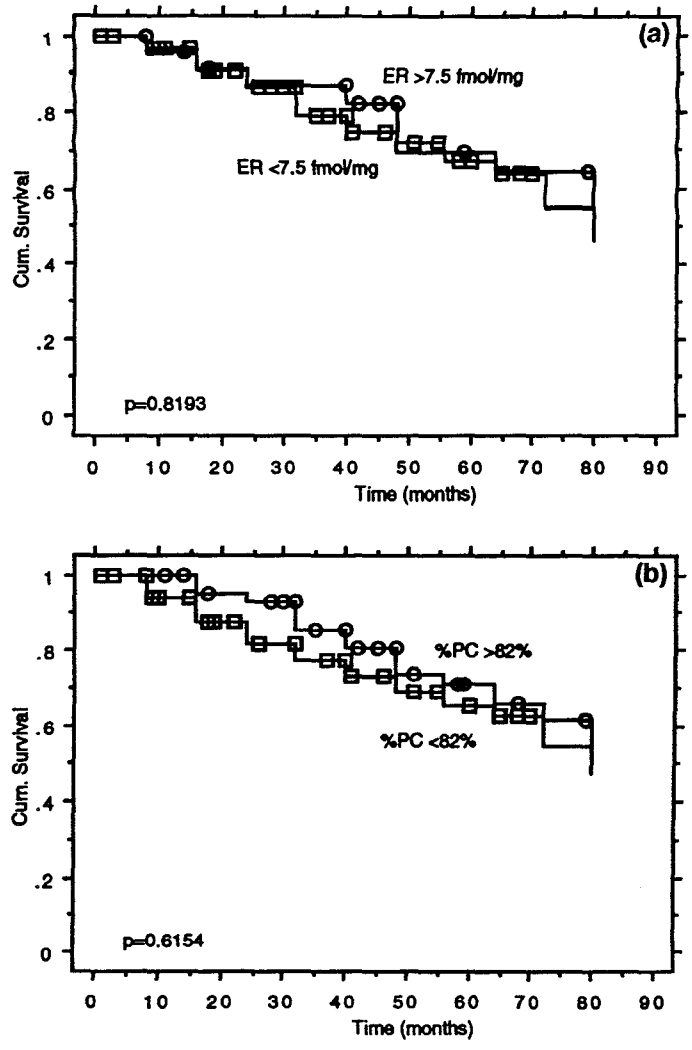


Figure 6. Overall survival of patients with invasive breast cancer according to ER status, as determined by the dextran-coated charcoal assay (DCCA) (A), and formalin/paraffin ERICA evaluated by %PC (B).

(30,31). ER value may also be influenced by the amount of protein in the homogenate contributed by noncancerous cells in the stroma. Because of these drawbacks, alternative methods for ER determination have been developed utilizing ER immunocytochemistry (ERICA). The advantage of this technique, at least theoretically, is that it allows direct visualization and thus direct ER determination in cancer cells. Because the quantity of ER in breast cancer is prognostically significant, a method for quantitating ER in tissue sections stained with ERICA, the HSCORE, has been devised (8–15). Although both components of the formula—staining intensity and percentage of positive cells—are subjective, it is the intensity of staining that is the most variable and subjective of the two. Although most studies evaluating the prognostic value of ERICA have uti-

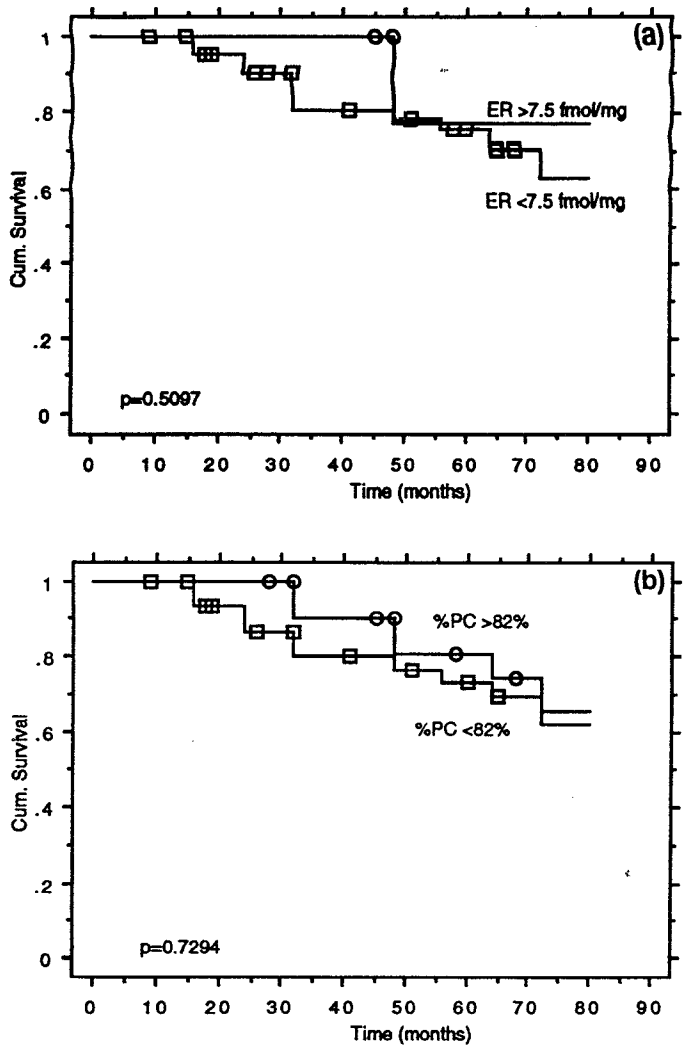


Figure 7. Overall survival of patients with node-negative invasive breast cancer according to ER status, as determined by the dextran-coated charcoal assay (DCCA) (A), and formalin/paraffin ERICA evaluated by %PC (B).

lized the HSCORE, a few reports have shown that the percentage of ER-positive cancer cells (%PC) is a significant prognostic indicator in women with breast cancer (17,18). If this is validated, then ERICA's prognostic value could rely solely on the %PC, thus eliminating the subjectivity associated with the assessment of staining intensity.

Our results show excellent correlation between %PC and HSCORE (Fig. 2), indicating that evaluation of %PC may be sufficient and may replace the HSCORE. Survival analysis confirmed this assumption and showed %PC to be a significant prognostic indicator in patients with breast cancer (Figs. 3C and 4C), giving results similar to those obtained with HSCORE (Figs. 3B and 4B). This supports the hypothesis that

the %PC, which is simple, less subjective, and subject to less variability, could replace HSCORE in determining ER as a prognostic marker in breast cancer.

The cutoff value (82%) for %PC, and similar values reported by others using frozen tissue sections (17,18), are substantially higher than those reported in studies using formalin-fixed and paraffin-embedded tissues. This value varies from a high of 30% (32) to as low as "any degree of specific nuclear staining recognizable above control" (33). The reason for this wide discrepancy could be attributed in part to the difference in antibodies used, but it is most likely due to the fact that formalin fixation reduces ER immunoreactivity substantially compared to fixatives like Zamboni's and is adversely affected by the time of fixation (34). Leong and Milios found that the percentage of ER-positive cells in archival breast cancer tissue was substantially lower than that in frozen tissues (35). From the biological standpoint, if in fact loss of ER by cancer cells is a sign of "bad intentions," one would expect the tumor to behave in a more aggressive fashion before most of the cells lose their ER. Recent publications, utilizing new anti-ER antibodies such as 1D5, and pretreatment of the sections with microwave irradiation in citrate buffer, have shown an increase in the sensitivity of assay in formalin-fixed and paraffin-embedded tissue (35). In our study, using this antibody and antigen retrieval method, the least p value was obtained with a high %PC value (89.5%), although the result was not significant. Another possible explanation for the wide variation is reported cutoff values for %PC is that most breast cancers would fall into one of two categories: less than 10% of the cancer cells positive for ER, or more than 80% of the cells positive for ER (83% of the frozen tumors) (Fig. 1). Since relatively few patients will have %PC values between 10% and 80%, it is easy to see that in most situations the cutoff value is going to be close to either 10% or 80% (depending on prognosis of patients with ER values between 10% and 80%).

Despite the theoretical problems of sampling attributed to the DCCA, ER status as measured by this method was a significant prognostic indicator in our hands (Figs. 3A and 4A), as has been reported by others. Because there is a wealth of published data relating ER DCCA to the response to hormonal therapy, and until ERICA shows similar value, we recommend that DCCA be used for evaluation of ER status if the tumor size is sufficient. However, in small tumors, %PC as determined by ERICA may be used instead of the DCCA and HSCORE in determining ER status. Additional studies are needed to determine whether %PC has a role in predicting the response of breast cancer to hormonal therapy.

With the advent of antigen retrieval, which makes ERICA on formalin-fixed and paraffin-embedded breast cancers possible, we tested the hypothesis that %PC is a significant predictor of survival in patients with breast cancer, when evaluated by paraffin section ERICA. The immunostained formalin/paraffin tissue sections showed a similar distribution of %PC in breast cancers (Fig. 5) to that seen with frozen sections (Fig. 1). This indicates that, indeed, most breast cancers will have $< 10\%$ or $> 80\%$ ER-positive cells (89% of formalin/paraffin tumors). Unlike frozen sections, a %PC of 82% was not a significant prognostic indicator in paraffin section ERICA (Figs. 6B and 7B). Initially, we thought that this disappointing result may have resulted from differences in the techniques used. However, we were surprised and somewhat relieved to find that ER, as determined by the DCCA on the same tumors, also had no significant prognostic value with a cutoff of 7.5 fmol/mg (Figs. 6A and 7A), in contrast to the frozen tumors group.

One of the reasons we used different populations of patients for the frozen and

formalin/paraffin studies was to test the hypothesis that our %PC cutoff value of 82% can be applied with confidence and is reproducible if used by others, especially in patients with node-negative breast cancer, a subset of breast cancer patients in whom ER and other prognostic markers are often evaluated. However, our data show that this is not the case. More interestingly, however, is the fact that ER, as determined by the DCCA, had no consistent prognostic value when performed on two separate groups of patients, even when only node-negative cancers are considered. Although it is easy to assume that the different reports in the literature regarding the prognostic value of ER are likely to be due to differences in methodologies and techniques used by different laboratories, our DCCA results were done in the same laboratory and nevertheless resulted in inconsistent results when the same cutoff value was used.

One explanation is that our two groups of patients with node-negative tumors were not similar in other important characteristics that are known to influence survival. Although not statistically significant, the frozen group tended to have slightly more moderately to poorly differentiated tumors and slightly more tumors with moderate to high nuclear pleomorphism than the formalin/paraffin group. The difference in size between the two groups was, however, significant. Although the size was not available on some tumors, the available information show that the tumors in the frozen group tended to be larger than those in the formalin/paraffin group ($p = 0.0054$). These taken together show that although the tumors in the two groups were stratified based on nodal status, other important differences that are known to influence survival still existed in both groups.

Based on our findings, we believe that evaluating the prognostic value of ER in node-negative patients is of no practical value when considered alone and that future studies should evaluate receptor status in more homogeneous patient populations, after taking into account other important traditional prognostic factors, such as tumor size, grade, and proliferative activity, in addition to the lymph node status. Because ER-DCCA was a significant prognostic indicator at a cutoff value of 7.5 fmol/mg in the frozen group and at a cutoff value of 25 fmol/mg in the formalin/paraffin group, we speculate that ER, and most likely other prognostic markers, will have different cutoff values in each well-characterized homogeneous patient population. Such studies will likely end the often confusing and contradicting results regarding potential prognostic markers and cutoff values.

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Transforming Growth Factor Alpha (TGF-α) Expression in Biopsies of Colorectal Carcinoma is a Significant Prognostic Indicator*

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Abstract. *Background: Predicting the outcome of patients with colorectal adenocarcinoma (CRCA) prior to surgery would be valuable in selecting high-risk individuals who may benefit from pre-operative adjuvant therapy. The aim of this study is to determine whether the expression of TGF-α in preoperative biopsies of patients with CRCA constitutes a significant prognostic indicator. Methods: We studied the expression of TGF-α in preoperative biopsies of 106 patients with CRCA, who had at least 5 years follow-up, using an anti-TGF-α monoclonal antibody and utilizing the ABC immunoperoxidase technique. For survival analysis, we used the actuarial survival method, and the Log Rank test for statistical significance. Results: CRCAs with low TGF-α expression (less than 25% of the tumor cells immunoreactive for TGF-α) had a significantly poorer survival than those with high TGF-α expression (more than 25%). After excluding from analysis biopsies showing mucinous or poorly differentiated CRCA, known predictors of poor prognosis, the results remained significant ($p=0.0289$). Conclusion: It is concluded, therefore, that low or absent expression of TGF-α in pre-operative biopsies of patients with CRCA, as detected by immunohistochemistry, is a significant predictor of an unfavorable outcome.*

Colorectal adenocarcinoma is the second most common malignancy in females and third in males. In 1989, 57,382 Americans died of this cancer, and it has been estimated that, in 1993, 152,000 new cases of colorectal cancer would be diagnosed and 57,000 deaths would be caused by it (1). Despite significant advances in surgery, as well as in radiation and chemotherapy, the mortality of colorectal carcinoma has remained steady over the past 40 years (2), although recent reports show a decrease in the incidence and mortality of rectal carcinoma attributed to surveillance and early detection (3). In order to make an impact on the mortality from this cancer, it has been recommended that research efforts include the development of indicators of the biological activity of these tumors in order to improve the pre-and post-operative staging (4). Such improved staging would result in better and more accurate selection of patients for the different available therapeutic modalities, based on their risk to benefit ratio.

Recently, more attention is being paid to neo-adjuvant (induction) therapy, which has shown promising results in advanced breast (5,6) and bladder (7) cancer. In contrast, clinical trials on colorectal carcinoma patients have yielded conflicting results, perhaps as a result of the inability to identify pre-operatively those patients with advanced tumors who are likely to benefit from such therapy (8). Similarly, failure to accurately predict the extent of some rectal cancers preoperatively has been blamed for the failures occasionally seen in conservative surgery such as transanal resection (9,10). Having a reliable predictor of outcome of patients with colorectal carcinoma should be of great help to treating physicians in choosing the best of available therapeutic options based on the pre-operative biopsy. This will be also helpful in selecting the most appropriate course of action for

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Key Words: Preoperative biopsy, survival, colon cancer, immunohistochemistry, TGF-α.

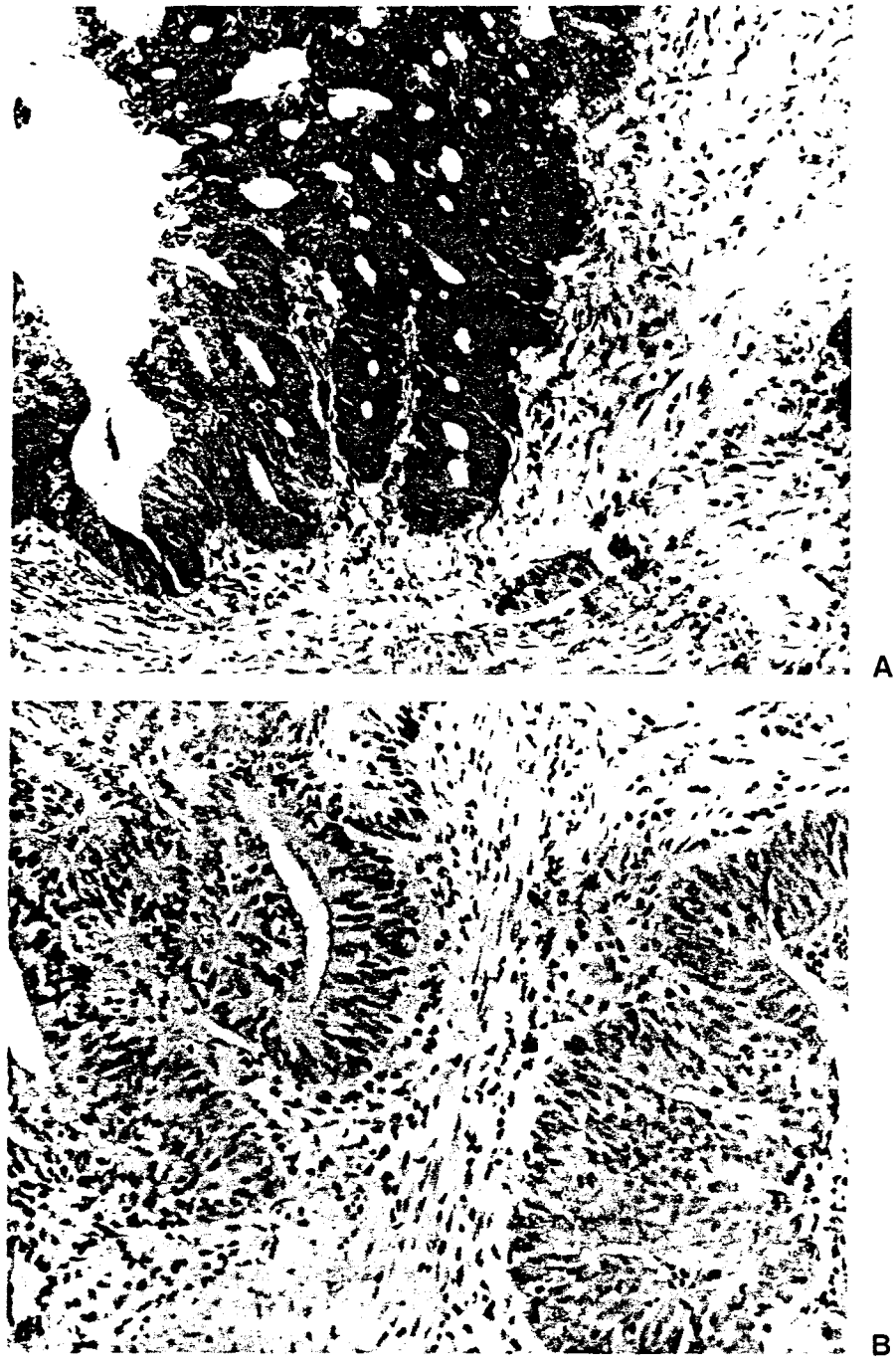

Figure 1. *Immunohistochemical staining for TGF-α in biopsies of colorectal adenocarcinomas, showing typical cytoplasmic staining. A, positive biopsy. B, negative biopsy. Immunoperoxidase staining, counterstained with hematoxylin (X 200).*

elderly patients with a biopsy diagnosis of colon cancer, in whom surgery caries a significant morbidity and mortality (11).

Transforming growth factor-Alpha (TGF-α) is a polypeptide that produces a mitogenic effect through interaction with the epidermal growth factor receptor (12), and is belived to promote carcinogenesis in the liver (13-16). TGF-α is secreted by many colon cancer cell lines (17-20), and was found to promote the growth of colon cancer cells in vitro (21-23), acting as an autocrine growth factor. The aim of

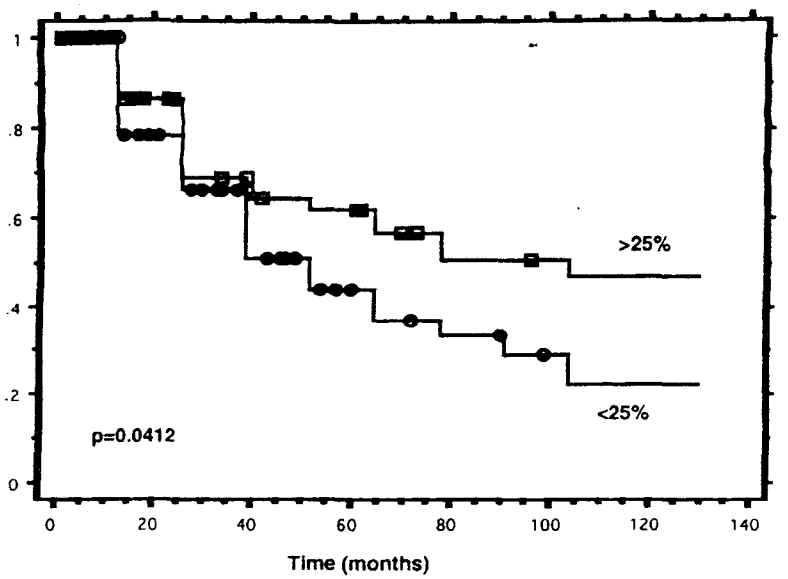

Figure 2. *Overall survival for all patients with colorectal adenocarcinoma, according to the percentage of TGF-α-positive cancer cells in the pre-operative biopsies.*

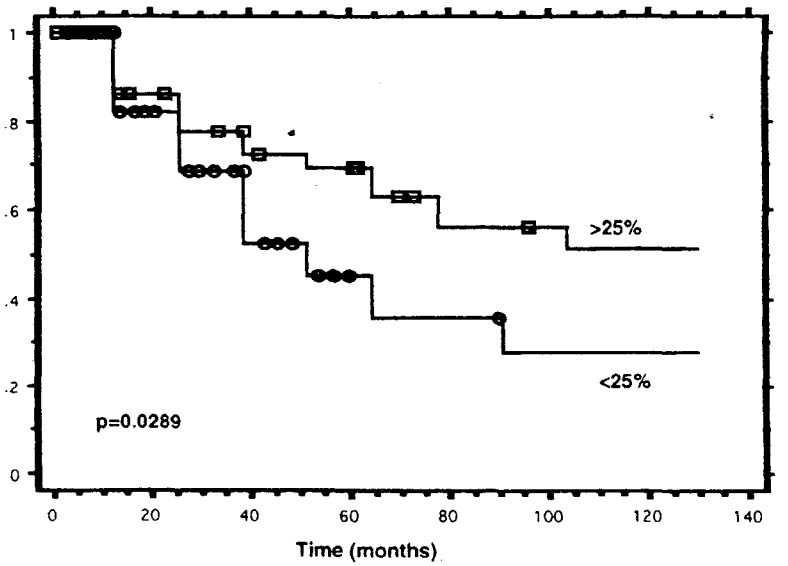

Figure 3. *Overall survival for patients with colorectal adenocarcinoma, excluding those with biopsies showing mucinous or poorly differentiated carcinoma, according to the percentage of TGF-α-positive cancer cells in the pre-operative biopsies.*

this work is to determine to what degree TGF-α expression in preoperative biopsies of colorectal carcinomas correlates with the aggressiveness of these cancers, and thus can be used as a predictor of patient outcome following surgery.

Materials and Methods

Patients. A total of 105 patients with colorectal adenocarcinoma, diagnosed and treated at The Methodist Hospital (TMH) in Houston, TX during the years1984-1989, were entered in the study. All of these patients were treated with surgery alone. The mean follow-up was 80 months, and the median 69 months. Follow up information was obtained from the Cancer Registry at TMH.

Histology. Hematoxylin and eosin-stained sections of preoperative biopsies were reviewed and evaluated for mucin production and histologic differentiation.

Immunohistochemistry. Sections of formalin-fixed and paraffin-embedded biopsies were cut and mounted on Fisher Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), and heated at 58°C for 4 hours. Sections were deparaffinized in xylene and rehydrated through decreasing concentrations of alcohol ending in PBS. Sections were then incubated with 0.05% saponin (Sigma Chemical Company, St Louis, MO) for 30 min at room temperature (RT), and washed with PBS X3. The sections were incubated with 2% normal horse serum in 1% BSA/PBS for 30 minutes at RT, washed in PBS, and incubated with anti TGF-α antibody (Ab-2, Oncogene Science, Uniondale, NY) diluted 1:50 in 0.1% BSA in PBS overnight at 4°C. The sections were washed in PBS, and the bound antibody was detected using Vectastain Elite ABC mouse kit (Vector Laboratories, Burlingame, CA), and DAB as the chromogen. Sections were counterstained with hematoxylin, dehydrated, and coverslipped using Accumount (Fisher) as the mounting medium. Slides incubated with 0.1% BSA in PBS instead of the primary antibody were used as negative control.

Evaluation of the immunostaining. The percent of cancer cells which stained for TGF-α was semiquantitatively scored as a) 0 (negative), b) <10%, c) 10-25%, d) 25-50%, e) 50-75%, or f) >75%. A cut-off value of 25% was reached by analyzing the data using all above scores as cut-off values. The cut-off value 25% produced the most significant result (least p value) by the log rank test.

Survival analysis. Was performed by the actuarial survival method and the log rank test for statistical significance, using StatView statistical software for the Macintosh with Survival Tools, version 4.5.

Results

Positive TGF-α staining was present in the cytoplasm of the tumor cells. The number of positive cells varied, and so did the intensity of staining. In all cases, when an area of the tumor was positive, all cells in that area were positive. Normal epithelial cells, when present in the same biopsy, also showed positive cytoplasmic staining for TGF-α. Examples of a positive and a negative case are shown in Figure 1.

When all carcinomas were considered in the analysis, cases with less than 25% of cancer cells positive for TGF-α were associated with worse outcome than those with >25% TGF-α-positive cells (p= 0.0412) (Figure 2). When poorly differentiated carcinomas and mucinous carcinomas were excluded from the analysis, the difference in survival remained significant (p= 0.0289) (Figure 3). No association was found between the percent of TGF-α positive cancer cells in the biopsy and the stage of the corresponding resected tumors.

Discussion

Because of the reported effects of TGF-α as a promoter of growth of colon cancer cells *in vitro* (21-23), it appeared somewhat paradoxical to find that decreased TGF-α expression is actually associated with a poorer survival rate. However, Markowitz *at al.* showed that TGF-α and the epidermal growth factor receptor are co-expressed in normal colonic epithelium as well as colonic adenomas, and they concluded that TGFa is an important physiologic stimulant of normal epithelial proliferation (24). Moreover, it has been recently shown that TGF-α can enhance the differentiation of a colon cancer cell line grown in 3-dimentional collagen gel (25), and that induction of terminal differentiation of human colon carcinoma cells is associated with a 20-fold induction of TGF-α (26). These findings seem to indicate that TGF-α is the physiologic growth and differentiation stimulator of the normal colon epithelium. Therefore, perhaps the loss TGF-α indicates loss of control over the physiologic growth and differentiation mechanism, and this is probably the reason why its absence is associated with a poor prognosis. This is similar to the poor outcome associated with the loss of estrogen receptors in breast cancer, while estrogen interaction with these receptors promotes growth of normal breast epithelial cells (physiologic growth promoter), as well as breast cancer cell lines *in vitro*.

Our results show that patients with colorectal carcinoma whose preoperative biopsy shows <25% of the cancer cells to be positive for TGF-α are likely to have a significantly worse prognosis than those with a biopsy containing >25% TGF-α-positive cells (p=0.0289). We conclude that the use of molecular prognostic markers, such as immunostaining for TGF-α, may be very useful in predicting the outcome of patients with colorectal carcinoma pre-operatively and therefore selecting the appropriate therapy, and in planning further clinical trials with neo-adjuvant therapy.

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