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Report

Quantitative analysis of mutant p53 protein in breast tumor cytosols and study of its association with other biochemical prognostic indicators in breast cancer

Michael A. Levesque,[1] Eleftherios P. Diamandis,[1] He Yu[1], and Donald J.A. Sutherland[2]
[1] *Department of Clinical Biochemistry, Toronto Western Division, The Toronto Hospital, 399 Bathurst Street, Toronto, Ontario M5T 2S8, Canada, and Department of Clinical Biochemistry, University of Toronto, 100 College Street, Toronto, Ontario M5G 1L5, Canada;* [2] *Toronto Bayview Regional Cancer Centre, Sunnybrook Health Science Centre, University of Toronto, 2075 Bayview Avenue, Toronto, Ontario M4N 3M5, Canada*

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Abstract

Breast tumors are thought to originate, grow, and metastasize in an environment which includes steroid hormone receptors, their cognate steroid ligands, and many gene products which are regulated by steroid hormone receptor-ligand complexes. In this paper we describe highly sensitive and quantitative immunofluorometric procedures for measuring three proteins that are candidate prognostic indicators in breast cancer, namely, the p53 tumor suppressor gene product, carcinoembryonic antigen (CEA), and prostate specific antigen (PSA). These proteins were quantified in over 950 cytosolic tumor extracts along with estrogen and progesterone receptors (ER, PR). Association analysis between all five biochemical parameters revealed strong negative associations between p53 and receptors and strong positive associations between CEA and receptors. Negative associations between p53 and CEA and between CEA and PSA were also found. These associations, not quantitatively studied in previous reports, are related to each other using a hypothetical model. The observed associations may further contribute to the understanding of the biology of breast tumors.

Introduction

Affecting approximately one woman in eight, breast cancer is the most common malignancy of women in North America. Although the incidence of this malignancy has been increasing steadily over the past 50 years [1], the mortality rates have held constant over this interval, owing to the earlier detection of smaller, premalignant lesions. After surgical removal of their primary tumors, a large number of patients will relapse, prediction of which may often be provided by well established pathological findings such as the presence and extent of axillary lymph node metastases [2]. The decision whether or not to treat each patient with toxic chemotherapeutic drugs or radiotherapy rests largely on the criterion of lymph node involvement [3]. However, a proportion of axillary node-negative patients will also relapse and this creates the requirement for additional prognostic factors that can predict, independent of node status, poor patient outcome and hence provide an indication for aggressive adjuvant therapy [4].

Although many features of the tumor or circulat-

Address for offprints: E.P. Diamandis, Dept. of Clinical Biochemistry, The Toronto Hospital, Toronto Western Division, 399 Bathurst Street, Toronto, Ontario M5T 2S8, Canada

ing tumor markers have been proposed as prognostic indicators, it is likely that combinations of them would be most useful [5]. Traditional breast cancer prognostic variables include: tumor size [2]; proliferation rate measured by flow cytometric determination of the S-phase fraction [6, 7]; DNA ploidy [8]; histologic and nuclear grades [9, 10]; and status of estrogen and progesterone receptors [11–13]. Other factors gaining acceptance are the tumor levels of the lysosomal enzyme cathepsin D [14, 15] and evidence of amplification or overexpression of the erbB2/neu oncogene [16, 17]. Newer promising factors whose potential merits are still being studied are the stress response proteins [18]; the anti-metastatic factor nm23 [19]; inducers of angiogenesis [20]; and many others. Included among this latter group is the much touted p53 protein, first associated with breast cancer in 1982 [21] and encoded by the most frequently altered gene in human cancer [22–24].

A body of evidence indicates that the chromosome 17p13-located p53 gene is a tumor suppressor gene [25, 26], whose loss or inactivation, primarily by missense mutations in evolutionarily conserved regions, coupled with loss of the remaining allele [27], permits transformed cells to escape normal proliferative constraints. The action of the expressed 53 kDa nuclear phosphoprotein remains unclear, although roles in control of DNA replication [28], and transcriptional activation [29] leading to growth arrest [30] or programmed cell death after DNA damage [31], have been postulated. Due to the short half-life of wild-type p53 (6–20 minutes), its levels are relatively low in normal cells. Mutant forms are stabilized [32], may form complexes with wild-type p53 [33], and are readily detectable by immunochemical methods. It is the largely intranuclear accumulation of the conformationally altered, nonfunctional p53 protein that reflects p53 gene point mutation [34], the consequences of which, in terms of tumor behaviour, have been the subject of intense investigation.

It became apparent that p53 abnormalities were implicated in breast carcinoma after cytogenetic studies reported allelic losses at 17p [35], after mutations in the p53 gene were shown to occur in 13–15% of primary breast tumors [36], and after accumulation of p53 protein was detected in 20–50% of malignant breast lesions [37]. Translation of the p53 accumulation pattern into information about the patient's disease, particularly prognosis, has been attempted in numerous studies with often disparate results. Immunohistochemically determined p53 overexpression has been associated with established indicators of poor prognosis such as: low levels of estrogen receptors [38–40] or of progesterone receptors [41]; high tumor grade [39, 42, 43]; metastatic spread [41]; nodal involvement [40]; neu oncogene expression [43, 44]; aneuploidy [44]; and high mitotic rate and proliferation index measured by flow cytometry [45]. Furthermore, although some studies did not reveal p53 accumulation to be an independent predictor of disease outcome [39, 45], others have shown association between p53 expression and shorter disease free and overall survival [42, 46, 47].

Variations in the findings between these studies may be accounted for, in part, by patient population heterogeneity, by differences in the fixation techniques, the anti-p53 antibodies employed, and by the rather subjective nature of the different systems used for scoring immunohistochemical staining patterns [48]. While not offering the same degree of subcellular antigen localization inherent in immunohistochemical techniques, immunoassay methods have the potential to generate quantitative values for p53 that are amenable to statistical analysis. To date, only reports by two other groups [49, 50] and by our own laboratory [51] have described the application of p53 immunoassays for the analysis of biological fluids including serum, cell line lysates, and tumor tissue extracts. Using an immunofluorometric procedure, we have demonstrated that 24% of breast tissue cytosols, prepared for steroid hormone receptor analysis, had elevated levels of p53 protein which were shown to be negatively correlated with both estrogen and progesterone receptors [51].

We are interested in studying the possible relationship between various biochemical parameters which have been found to be altered in tumor tissue. It is now generally accepted that steroid hormones and their receptors play a crucial role in breast tumor growth. Thus, study of the possible association

between various biochemical markers and receptors may help understand better the mechanisms of gene regulation by steroid hormone-receptor complexes. We here report on the quantitative analysis of p53 protein in the largest series of breast tumors reported to date (over 950 samples) and on the association between p53 levels and levels of steroid hormone receptors, CEA, and prostate specific antigen (PSA), a new potential biochemical prognostic marker in breast cancer. Our studies have revealed a strong negative association between p53 and receptors and a strong positive association between CEA and receptors [51–53]. However, negative associations between CEA and p53 were also found along with negative associations between CEA and PSA. We found evidence that progestins may be involved in the regulation of p53 gene repression and that estrogens alone may be involved in the regulation of the CEA gene derepression in breast cancer. Based on these findings, we propose a model which explains the interrelationships among the two steroid hormone receptors, steroids, p53, CEA, and PSA. Our model may be used as an aid in devising prognostic panels in breast cancer.

Materials and methods

Patients

The patient population consisted of 965 females with primary breast carcinoma. Tumor specimens were obtained by surgical resection for steroid hormone receptor quantification, at 58 Ontario and two New Brunswick hospitals, between October 1992 and March 1993.

Cytosol preparation

A representative sample of each resected tumor was snap-frozen using liquid nitrogen or dry ice, according to standard practice [54], and transported in dry ice or liquid nitrogen to Sunnybrook Health Science Centre. After no more than five days of storage at – 70° C, approximately 0.5 g of tissue was immersed in liquid nitrogen, manually pulverized to a fine powder, and homogenized in 10 mL of 10 mM Tris buffer, pH 7.40, containing 1.5 mM EDTA and 5 mM sodium molybdate, with a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY 11590) using a single 5 sec burst at setting 6. Correspondingly less buffer was used if the tissue had a mass less than 0.5 g. Because of the heat-lability of the receptor proteins, all materials and reagents in contact with the tissue or homogenate were chilled on ice. Separation of the cellular fractions, performed by ultracentrifugation at 105,000 g, at 4° C, for one hour with a Beckman ultracentrifuge (Beckman Instruments Inc., Fullterton, CA 92634), yielded a cytosol fraction which was carefully collected and immediately assayed for total protein using the Lowry method [55] and for estrogen and progesterone receptors. The tumor extracts were subsequently analyzed for p53, CEA, and PSA levels after transport to the Toronto Hospital, Western Division, and storage at – 70° C for not more than one month. These biochemical parameters were found to be stable at – 70° C for at least two months.

Hormone receptor assays

The estrogen and progesterone receptor concentrations of the cytosols were determined using enzyme immunoassay kits (Abbott Laboratories, North Chicago, IL 60064) which employ double monoclonal antibodies to each receptor [56–58]. For each assay, the methods described in the package inserts were followed. Specimens with values > 10 fmol/mg cytosol protein were considered positive as suggested by others [46].

Immunofluorometric assay of p53

The coating buffer was a 50 mmol/L Tris, pH 7.40, containing 0.5 g/L NaN_3. The wash solution was a 5 mmol/L Tris buffer, pH 7.80, containing 150 mmol/L NaCl, and 0.5 g/L Tween 20. The blocking solution was a 50 mmol/L Tris buffer, pH 7.80, containing 10 g/L bovine serum albumin (BSA) and 0.5 g/L NaN_3. The anti-p53 monoclonal antibody

PAb240 diluent was a 50 mmol/L Tris buffer, pH 7.80, containing 60 g/L BSA, 0.5 g/L NaN_3, and 0.5 mol/L KCl. The polyclonal CM-1 anti-p53 antibody diluent was a 50 mmol/L Tris buffer, pH 7.80, containing 60 g/L BSA and 0.5 g/L NaN_3. The diluent for the goat anti-rabbit antibody, conjugated to alkaline phosphatase (GARlg-ALP), was the same as the PAb240 antibody diluent. The stock enzyme substrate solution (diflunisal phosphate) was 0.01 mol/L in 0.1 mol/L NaOH. For the assay, the stock substrate solution was diluted 10-fold in the substrate buffer, a 0.1 mol/L Tris buffer, pH 9.10, containing 0.15 mol/L NaCl, 1 mmol/L $MgCl_2$, and 0.5 g/L NaN_3. The developing solution, containing 1 mol/L Tris base, 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$, and 3 mmol/L EDTA (no pH adjustment), was prepared as described elsewhere [59, 60].

A noncompetitive 'sandwich' immunoassay was developed for the measurement of mutant p53 protein concentration [51]. In brief, 96 well polystyrene microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) were incubated overnight at 4° C with 100 µL of goat anti-mouse immunoglobulin (1 mg/ml, Jackson ImmunoResearch, West Grove, PA 19390) diluted 400-fold in the coating buffer. After six washing cycles on an automatic plate washer (Adil Instruments, Strasbourg, France) the wells were blocked by the addition of 250 µL of blocking solution at least 30 minutes before the samples were added. Two cycles of washing were followed by addition of 50 µL of unknown, standard or control samples together with 100 µL of a 20-fold diluted PAb240 mouse monoclonal antibody [61] in the PAb240 diluent and incubation for 3 h at 37° C with shaking. This antibody is well-characterized [61], is specific for mutant p53, and was produced in-house from hybridoma cell culture supernatants (approximate antibody concentration 30 µg/mL). After six cycles of washing, polyclonal rabbit CM-1 antibody (Novocastra Laboratories, Newcastle upon Tyne, UK), raised against recombinant human wild-type p53 protein [62], was diluted 5,000-fold in the CM-1 diluent and added in 100 µL volumes to the wells for a one hour incubation at room temperature, the temperature of all subsequent incubations for this assay. The plates were washed again as above before 100 µL of goat anti-rabbit antibody conjugated to alkaline phosphatase (1 mg/mL, Jackson) diluted 5,000-fold in the GARlg-ALP diluent was added for another one hour incubation. The final six cycle washing step was followed by the addition of 100 µL of the diluted enzyme substrate and incubated for 10 minutes. Developing solution was added in 100 µL volumes without washing the wells for a one minute incubation. The fluorescence of the final solution was measured on the Cyberfluor-615 Immunoanalyzer, a time-resolved fluorometer (Cyberfluor Inc., Toronto, Ontario). Data reduction was automatic through the immunoanalyzer software.

Assay standardization and quality control

There is no standard p53 preparation available. In order to compare results in a quantitative fashion, we have identified and selected a breast tumor extract with high p53 concentration as the calibrating material. This sample was given an arbitrary concentration value of 1,000 units per liter (1,000 U/L). Several dilutions of this standard in a 50 mmol/L Tris buffer, pH 7.80, containing 60 g/L BSA and 0.5 g/L NaN_3, were prepared to give concentrations of 0, 2, 5, 20, 50, and 200 U/L. These solutions were used as primary standards for calculating the unknown concentrations. All samples were run in duplicate and samples with concentrations > 200 U/L were reassayed in dilution.

For quality control purposes, we chose six cytosols with concentrations between 2–200 U/L and assayed them in each run in order to monitor performance. Standards and quality control samples were stored in aliquots at – 70° C and thawed just before use.

CEA immunofluorometric assay

CEA was quantified in the breast tumor cytosols using a double-monoclonal immunoassay [63] modified by us in order to enhance sensitivity. The compositions of the coating buffer, wash solution, enzyme substrate, substrate buffer, and developing solution were identical to those described above for the p53 immunoassay. A monoclonal anti-CEA an-

tibody, 1 mg/mL, clone code 5911 (Medix Biochemica, Kauniainen, Finland), was diluted 200-fold in coating buffer and added to microtiter wells in 100 µL volumes for overnight incubation at 4° C. Six cycles of washing were followed by the addition, in duplicate, of 50 µL of each unknown, standard, or control, together with 50 µL of a 50 mmol/L Tris buffer, pH7.80, containing 60 g/L BSA and 0.5 g/L NaN_3 (buffer A), for one hour incubation at room temperature. After another six washes, 100 µL of a 1000-fold dilution in buffer A of a biotinylated monoclonal anti-CEA antibody, clone 5914 (also from Medix Biochemica), that recognizes a different CEA epitope, was added to all wells and allowed to incubate for one hour at room temperature followed by six washes. A streptavidin-alkaline phosphatase conjugate (1 mg/mL, Jackson ImmunoResearch) diluted 20,000-fold in buffer A was next added in 100 µL volumes and incubated for 15 minutes at room temperature. All subsequent steps were performed as in the p53 assay, namely: a final wash step; the incubation of wells with 100 µL of a 10-fold diluted enzyme substrate for 10 minutes; the addition of 100 µL of developing solution for one minute; and the time-resolved fluorescence measurement on the Cyberfluor-615 Immunoanalyzer.

The CEA standards of 100, 25, 5, 1, 0.1, and 0 µg/L were prepared by serial dilution of a 0.275 g/L concentrate solution (Scripps Laboratories, San Diego, CA 92103) in buffer A. Lyphocheck immunoassay human control sera, levels 1, 2, and 3 (Bio-Rad Laboratories, Clinical Division, Richmond, VA 94801) were assayed on each run for quality control purposes. Both standards and controls were aliquoted into small volumes and stored at – 70° C until use.

PSA assay

The PSA assay used was described in detail elsewhere [64].

Statistical analysis

Statistical calculations were performed using SAS software (SAS Institute, Cary, NC 27512). They included the determinations of Pearson correlation coefficients between the concentrations of p53, CEA, PSA, and steroid receptors, as well as the generation of contingency tables to examine the relationships between the biochemical parameters. Statistical significance was determined using chi-square tests, and $p < 0.05$ was considered significant throughout.

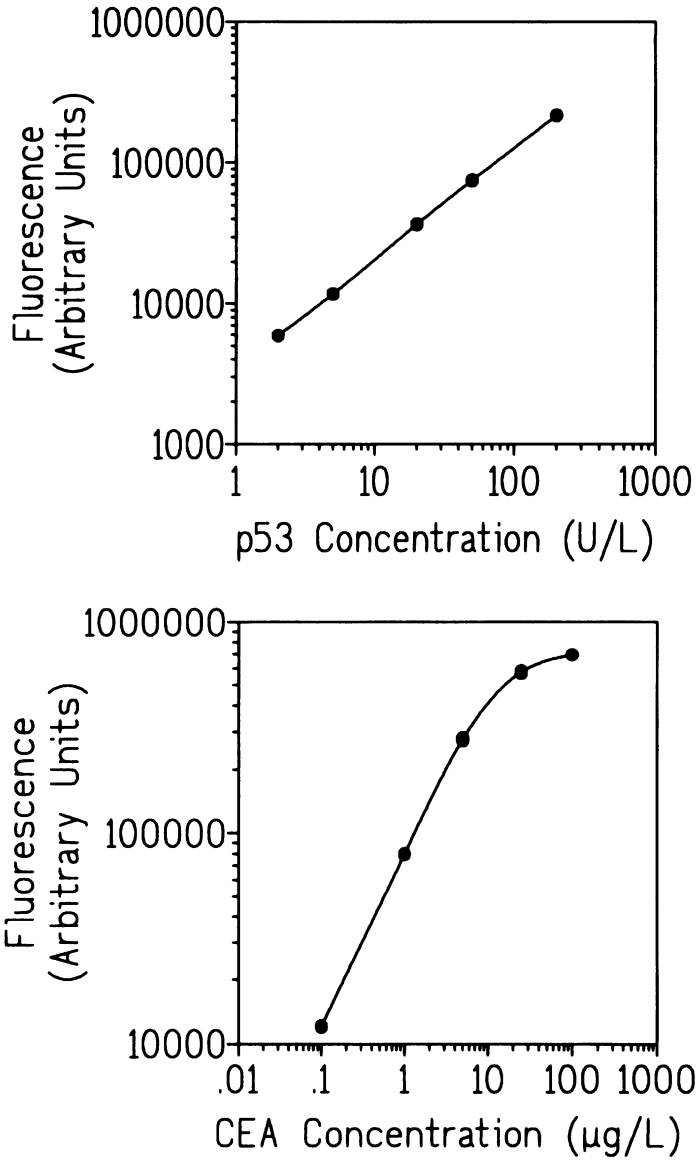

Fig. 1. Calibration curves of the p53 assay (upper panel) and CEA assay (lower panel). The fluorescence of the zero standard was approximately 1000 counts for both assays and was subtracted from all other measurements.

Table 1. Day-to-day precision of the p53 and CEA immunoassays

CEA (μg/L) or p53 (U/L)					
Control sample	Mean	Standard deviation	CV (%)	N[1]	Days[2]
CEA, A	1.51	0.16	10.8	6	20
B	9.12	0.94	10.2	6	20
C	33.9	8.2	24.3	6	20
p53, A	2.20	0.17	7.9	10	80
B	3.95	0.54	13.6	10	80
C	8.37	0.86	10.2	10	80
D	8.74	0.66	7.6	10	80
E	110	15.1	13.8	10	80
F	184	32	17.6	10	80

[1] Number of runs performed
[2] Testing period in days

Results

Biochemical data

We obtained 965 tumors for which we generated data for steroid hormone receptors (965 samples), quantitative p53 levels (956 samples), carcinoembryonic antigen (CEA) levels (953 samples), and PSA levels (504 samples).

Assay performance and frequency distributions

Typical calibration curves for the p53 assay and the CEA assay are shown in Fig. 1. The detection limit, defined as the concentration of p53 or CEA that could be distinguished from zero with 95% confidence, was found to be 0.2 U/L for p53 and 0.01 μg/L for the CEA assay. The day-to-day precision of the p53 assay and of the CEA assay are shown in Table 1. The vast majority of data in the literature regarding p53 levels in tumors are based on qualitative immunohistochemical investigations. We here clearly demonstrate the ability of immunological procedures to assess p53 levels in a quantitative and reproducible fashion, with day-to-day coefficients of variation of approximately 7–18% in the entire measuring range and over extended periods of time (80 days). The precision of the

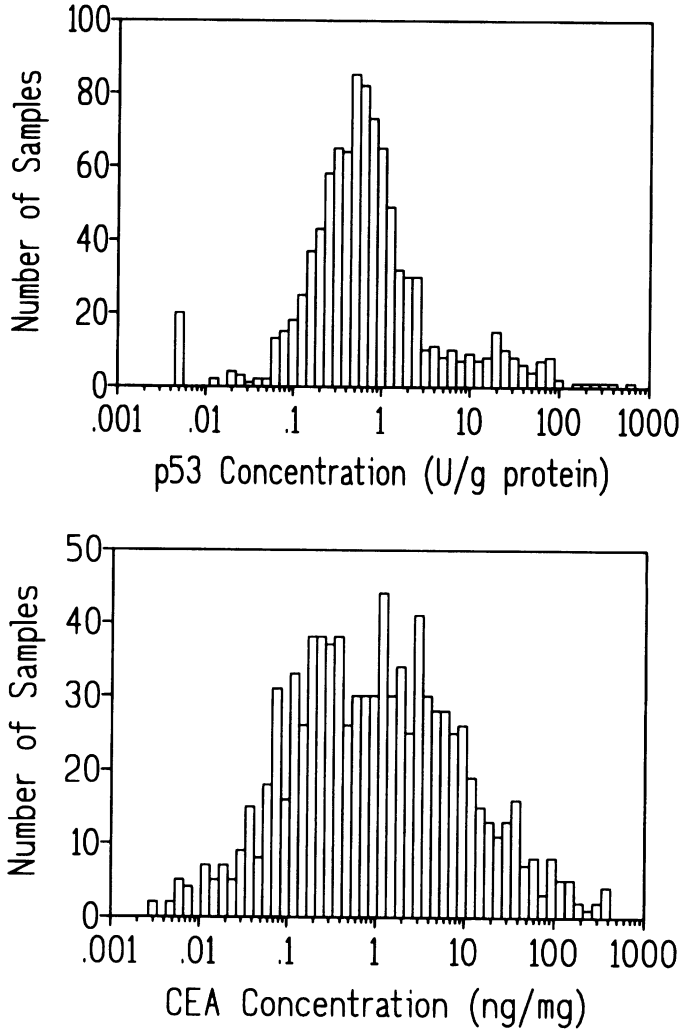

Fig. 2. Frequency distributions of p53 and CEA values in 956 or 953 breast tumor extracts, respectively. The x-axis is logarithmic in both plots.

Table 2. Statistical parameters for the distribution of p53 protein, CEA and steroid hormone receptors in breast tumor cytosols

Parameter	p53[1]	CEA[2]	ER[3]	PR[4]
Number of samples	956	953	965	965
Minimum	0	0	0	0
Maximum	795	472	979	1008
Mean	7.2	10.7	131	129
Standard deviation	41.4	39.2	152	175
Percentile 5th	0.078	0	0	0
10th	0.15	0.038	1	1
25th	0.31	0.18	8	3
50th	0.68	0.94	69	39
75th	1.49	4.82	216	216
90th	8.5	19.3	364	405
95th	29.7	46.8	438	473

[1] Values in U/g of total protein
[2] Values in ng/mg of total protein
[3] Estrogen receptor, values in fmol/mg of total protein
[4] Progesterone receptor, values in fmol/mg of total protein

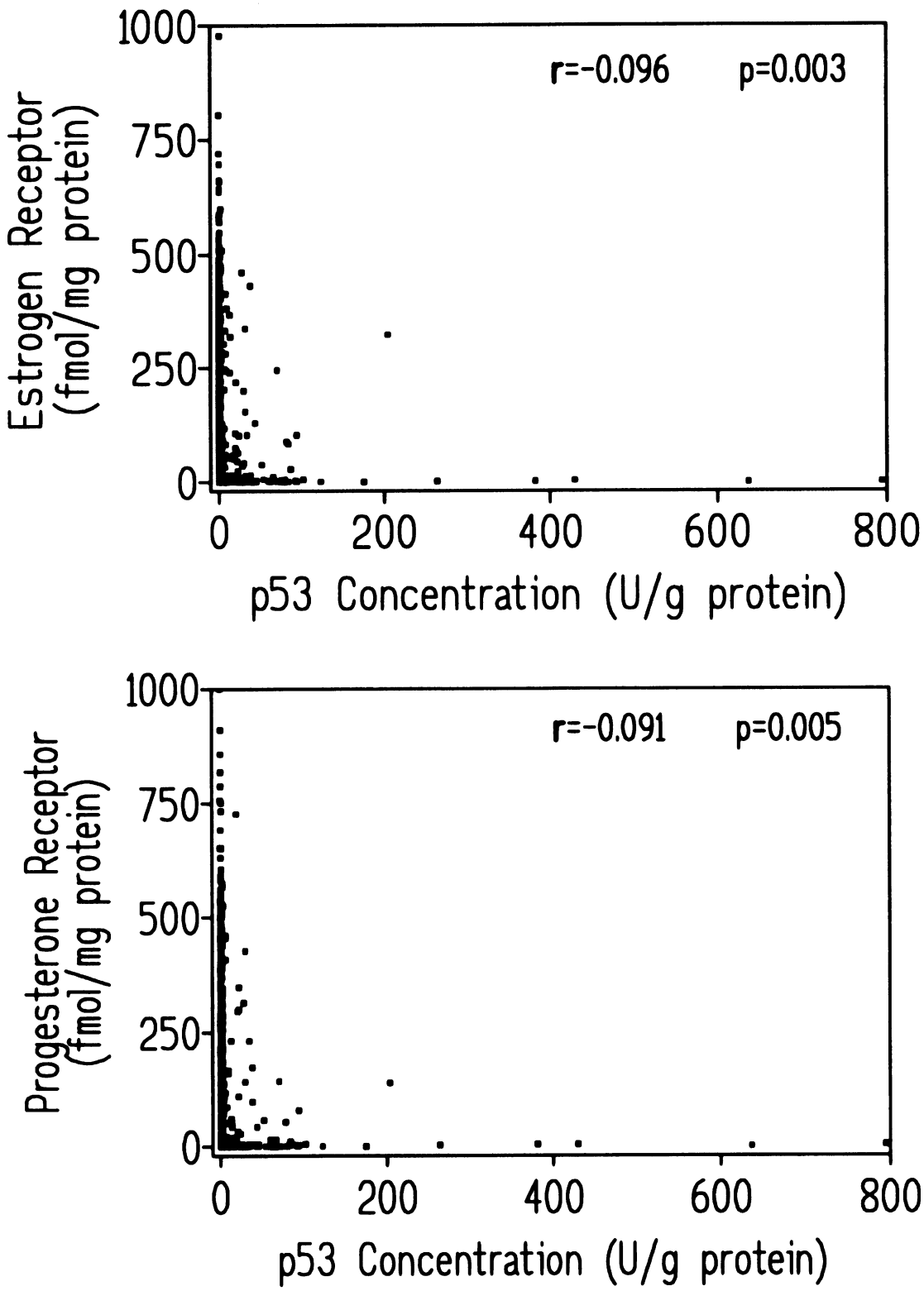

Fig. 3. Scatter diagrams of receptor vs. p53 levels in 965 breast tumor extracts. r is the Pearson correlation coefficient.

CEA assay is also shown in Table 1. Compared to commercially available CEA kits, our assay is at least 10-fold more sensitive, allowing accurate CEA assessment in the tumor extracts even when the analyte is present at relatively low concentrations.

In Fig. 2 we present the frequency distribution of p53 levels in 956 breast tumor cytosols, expressed as p53 units per gram of total protein, and the frequency distribution of CEA levels in 953 breast tumor cytosols, expressed as ng of CEA per mg of total protein. These data are expressed as units or ng of p53 or CEA, respectively, per mg of total protein in the cytosols in order to compensate for variations in tumor cell numbers extracted. Some statistical par-

Table 3. Relationship between p53 and estrogen or progesterone receptors

	Percentage of cases				Percentage of cases			
p53, U/g[1]	ER < 10[2]	ER ≥ 10	X^2	P	PR < 10	PR ≥ 10	X^2	P
< 1 (613)	21	79	18.8	< 0.001	32	68	15.8	< 0.001
≥ 1 (342)	34	66			45	55		
< 2 (759)	21	79	38.8	< 0.001	32	68	33.1	< 0.001
≥ 2 (196)	43	57			54	46		
< 3 (814)	22	78	48.2	< 0.001	33	67	33.7	< 0.001
≥ 3 (141)	50	50			58	42		
< 10 (864)	23	77	47.3	< 0.001	33	67	40.6	< 0.001
≥ 10 (91)	56	44			67	33		
< 20 (888)	24	76	38.9	< 0.001	34	66	29.3	< 0.001
≥ 20 (67)	58	42			67	33		
< 50 (927)	25	75	30.0	< 0.001	35	65	15.2	< 0.001
≥ 50 (28)	71	29			71	29		

[1] Number of samples in brackets

[2] ER, estrogen receptor; PR, progesterone receptor. Concentrations are in fmol/mg of total protein

Table 4. Relationship between p53 levels and combined receptor results

	Percentage of cases					
p53, U/g[1]	ER(+) PR(+)[2]	ER(+) PR(−)	ER(−) PR(+)	ER(−) PR(−)	X^2	P
< 1 (613)	65	13	3	19	21.9	< 0.001
≥ 1 (342)	51	15	4	30		
< 2 (759)	65	14	3	18	45.6	< 0.001
≥ 2 (196)	43	14	3	40		
< 3 (814)	64	14	3	19	51.9	< 0.001
≥ 3 (141)	38	13	4	45		
< 10 (864)	63	14	3	20	57.3	< 0.001
≥ 10 (91)	31	13	2	54		
< 20 (888)	63	14	3	20	44.4	< 0.001
≥ 20 (67)	30	12	3	55		
< 50 (927)	62	14	3	21	31.3	< 0.001
≥ 50 (28)	22	7	7	64		

[1] Number of samples in brackets

[2] ER, estrogen receptor; PR, progesterone receptor. Cutoff points were 10 fmol/mg of total protein

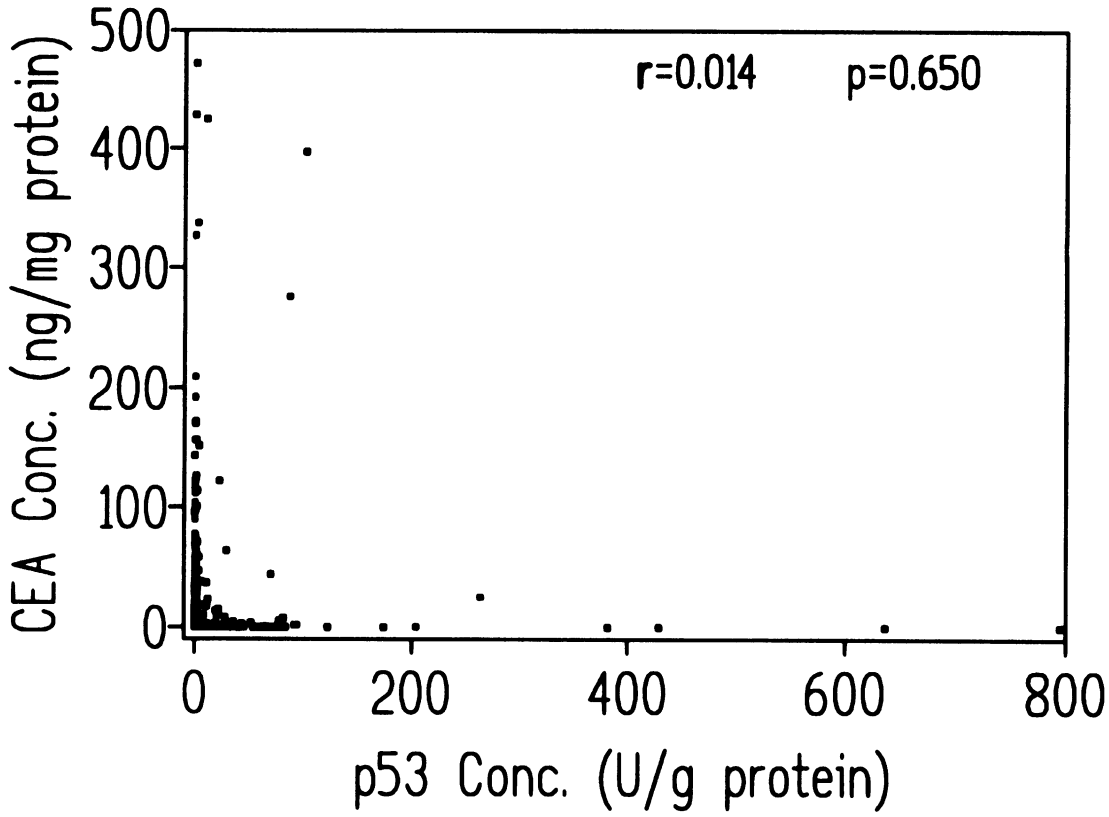

Fig. 4. Scatter diagram of CEA vs. p53 levels in 953 breast tumor extracts.

Table 5. Relationship between p53 and CEA concentrations at two cutoff levels of CEA

	Percentage of cases				Percentage of cases			
p53, U/g[1]	CEA < 0.1[2]	CEA ≥ 0.1	X^2	P	CEA < 0.2	CEA ≥ 0.2	X^2	P
< 1 (606)	16	84	2.76	0.096	24	76	3.40	0.065
≥ 1 (338)	20	80			30	70		
< 2 (748)	16	84	3.59	0.058	25	75	3.99	0.046
≥ 2 (196)	22	78			32	68		
< 3 (803)	16	84	9.08	0.003	24	76	7.60	0.006
≥ 3 (141)	26	74			35	65		
< 10 (853)	16	84	17.1	< 0.001	24	76	16.7	< 0.001
≥ 10 (91)	33	67			44	56		
< 20 (877)	16	84	14.4	< 0.001	25	75	11.1	0.001
≥ 20 (67)	34	66			43	57		
< 50 (916)	17	83	9.65	0.002	25	75	8.58	0.003
≥ 50 (28)	39	61			50	50		

[1] Number of samples in brackets

[2] CEA values in ng/mg of total protein

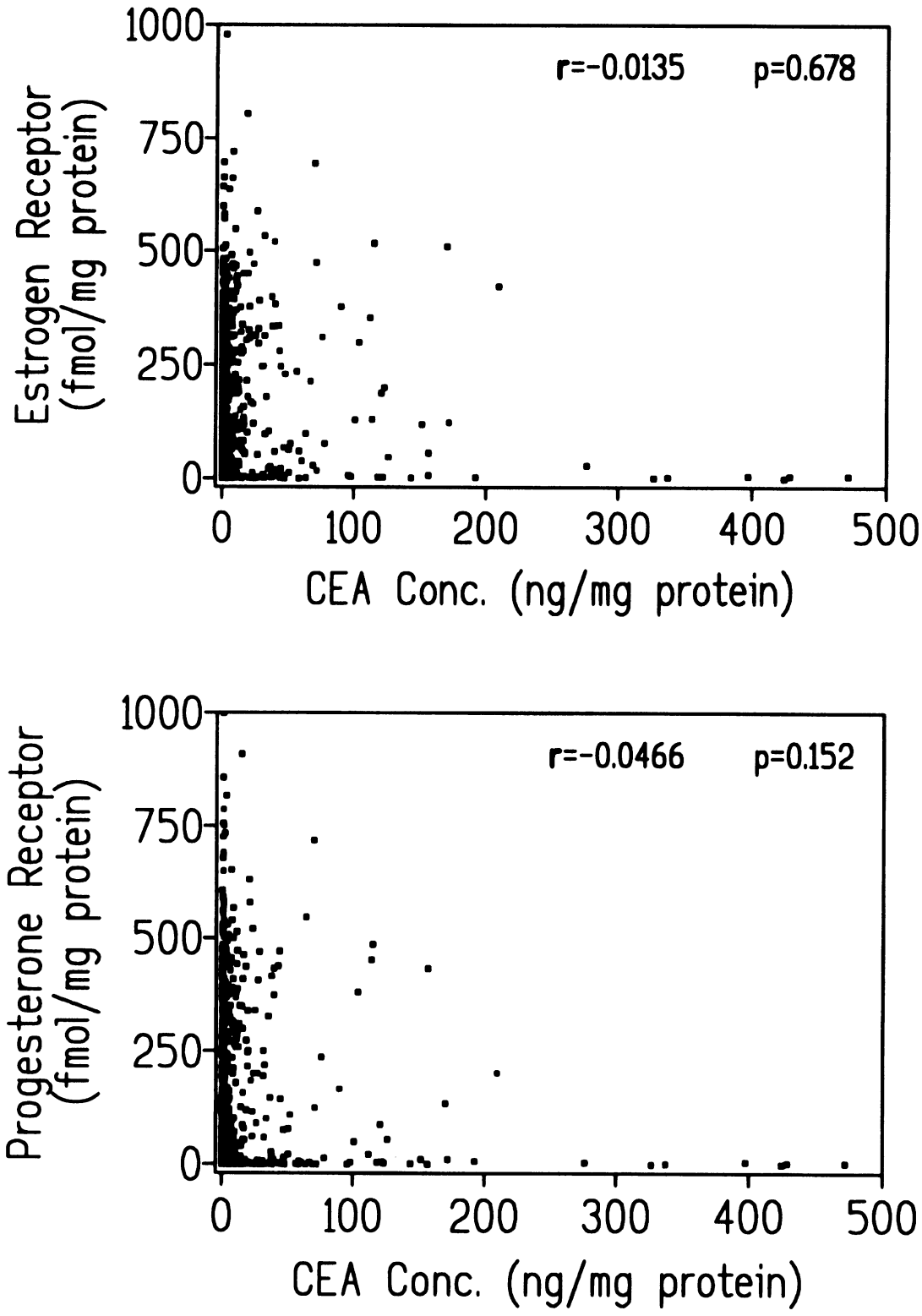

Fig. 5. Scatter diagrams of receptor vs. CEA levels in 953 breast tumor extracts.

ameters are also presented in Table 2 along with data for the steroid hormone receptors. The distribution of PSA levels in breast tumors has been described elsewhere [65].

Relationships between biochemical parameters

Linear regression analysis between p53 levels and estrogen receptor or progesterone receptor levels revealed a weak negative correlation. The Pearson correlation coefficients were $r = -0.096$, $P = 0.003$, and $r = -0.091$, $P = 0.005$, for estrogen and proges-

Table 6. Relationship between levels of CEA and steroid hormone receptors

	Percentage of cases				Percentage of cases			
CEA, ng/mg[1]	ER < 10[2]	ER ≥ 10	X^2	P	PR < 10	PR ≥ 10	X^2	P
< 0.1 (166)	54	46	84.5	< 0.001	60	40	47.6	< 0.001
≥ 0.1 (786)	20	80			31	69		
< 0.2 (251)	45	55	65.4	< 0.001	50	50	28.7	< 0.001
≥ 0.2 (701)	19	81			31	69		

[1] Number of samples in brackets

[2] ER, estrogen receptor; PR, progesterone receptor. Values in fmol/mg of total protein

terone receptors, respectively (Fig. 3). Although it is evident that many estrogen or progesterone receptor-positive tumors are p53-negative, and vice-versa, there are also tumors which are positive or negative for both biochemical parameters (receptors and p53). This is in support of our previous preliminary data [51] and data by others [46, 47] who suggest that p53 protein is an independent prognostic indicator in breast cancer, offering additional prognostic information than the routinely used receptors.

The relationship between receptors and p53 was further examined by association analysis using 2×2 contingency tables. For this analysis, we used the 10 fmol/mg of protein cutoff levels for the receptors [46]. The cutoff levels for p53 protein were arbitrary since no studies have been published which utilize quantitative p53 analysis except in rare instances [49–51]. We have thus adopted the practice of analyzing our data at various cutoff levels of p53. The quantitative association analysis is not feasible when the p53 levels are assessed qualitatively with immunohistochemical techniques.

The association analysis between p53 and steroid hormone receptor levels is presented in Table 3. There is a strong negative association between the receptors and p53 with highly significant P values (< 0.001) at any cutoff level of p53 studied, between 1–50 U/g. Clearly, the percentage of ER(−) tumors increases and the percentage of ER(+) tumors decreases as the p53 cutoff level is increased (Table 3). The same comments apply for the PR comparisons. Association analysis performed after combining the two receptors in four groups [i.e. ER(+) PR(+); ER(+) PR(−); ER(−) PR(+); ER(−) PR(−)] and three degrees of freedom, revealed similar results (Table 4). Interestingly, the group of tumors with only one of the two receptors being positive was not associated with the presence or absence of p53 at any level of the p53 cutoff values. Based on this new observation, we speculate that if the p53 gene is under the regulatory influences of the steroid hor-

Table 7. Relationship between CEA levels and combined receptor results

	Percentage of cases					
CEA, ng/mg[1]	ER(+) PR(+)[2]	ER(+) PR(−)	ER(−) PR(+)	ER(−) PR(−)	X^2	P
< 0.1 (166)	38	8	3	52	97.6	< 0.001
≥ 0.1 (786)	65	15	2	16		
< 0.2 (251)	47	8	3	42	73.0	< 0.001
≥ 0.2 (701)	66	15	3	16		

[1] Number of samples in brackets

[2] ER, estrogen receptor; PR, progesterone receptor. Cutoff points were 10 fmol/mg of total protein

mone receptors in breast cancer, it is likely repressed by mechanisms which involve both the ER and PR.

Although the concentrations of p53 and CEA in the breast tumor cytosols were not significantly linearly correlated (Fig. 4, r = 0.014, p = 0.65), association analysis with 2 × 2 contingency tables revealed that tumors positive for p53 were significantly associated with CEA-negative tumors, especially at cutoff levels of 0.1–0.2 ng/mg for CEA (Table 5). Such low CEA levels are usually unmeasurable by current immunological assays but easily and precisely measured by our ultrasensitive procedure. At higher CEA cutoff levels the association becomes weaker, because of the reclassification of many CEA-positive tumors into the category of CEA-negative tumors (data not shown). This association between p53 and CEA levels in breast tumors was not previously recognized.

Linear regression analysis between levels of CEA and steroid hormone receptors (Fig. 5) revealed no statistically significant correlations. However, association analysis using 2 × 2 contingency tables, revealed that tumors positive for CEA were strongly associated with positive estrogen and/or progesterone receptor status (Table 6). This association was very significant ($P < 0.001$) at any CEA cutoff level between 0.1–1 μg/mg of protein, but the highest X^2 values were observed with a CEA cutoff value of either 0.1 or 0.2 μg/mg protein. Reanalysis of the data for the four groups of receptor pairs [ER(+) PR(+); ER(+) PR(−); ER(−) PR(+); ER(−) PR(−)] and CEA cutoff levels of either 0.1 or 0.2 μg/mg gave the results of Table 7 which confirm the strong associations between CEA and ER and PR. However, these data also reveal that tumors positive only for ER also tend to be CEA-positive, suggesting that CEA production is under the control of the estrogen receptor alone. Tumors positive only for the PR are not associated with CEA. This suggestion is further supported by the data of Table 6, which reveal much stronger associations between CEA and estrogen receptors ($X^2 = 84.5$ or 65.4) in comparison to CEA and progesterone receptors ($X^2 = 47.6$ or 28.7). Based on the fact that the estrogen and progesterone receptor concentrations are also associated with each other, we propose that the association between CEA and progesterone receptors is indirect in nature (see below). The strong associations between CEA and receptors reported here have not been previously realized mainly because of the inability of CEA methods to detect very low levels of CEA in tumor extracts.

Table 8. Relationship between CEA and PSA levels in breast tumors

CEA, ng/mg [1]	Percentage of cases			
	PSA < 0.05 μg/L	PSA ≥ 0.05 μg/L	X^2	P
< 0.1 (97)	80	20	0.41	0.52
≥ 0.1 (407)	77	23		
< 0.2 (137)	79	21	0.080	0.78
≥ 0.2 (367)	78	22		
< 0.5 (223)	75	25	1.62	0.20
⩾ 0.5 (281)	80	20		
< 1 (267)	74	26	3.92	0.048
≥ 1 (237)	82	18		
< 3 (344)	75	25	4.55	0.033
≥ 3 (160)	84	16		
< 10 (425)	77	23	2.55	0.11
⩾ 10 (79)	85	15		

[1] Number of samples in parentheses

Linear regression analysis between levels of CEA and PSA in breast tumor cytosols revealed no significant correlation. Association analysis between CEA and PSA, for 504 tumor extracts, using a cutoff level of 0.05 μg/L of PSA as previously reported [65] and various cutoff levels of CEA, gave the results of Table 8. Clearly, there is no statistically significant association between PSA and CEA when the CEA cutoffs used were up to 0.5 ng/mg protein. When the CEA cutoffs were raised to either 1 or 3 ng/mg protein, there was a weak but statistically significant negative association (Table 8).

We have previously found that PSA-positive tumors are associated with positive estrogen and progesterone receptor status and that there is no association between p53 and PSA [65].

Based on the data presented here and previously

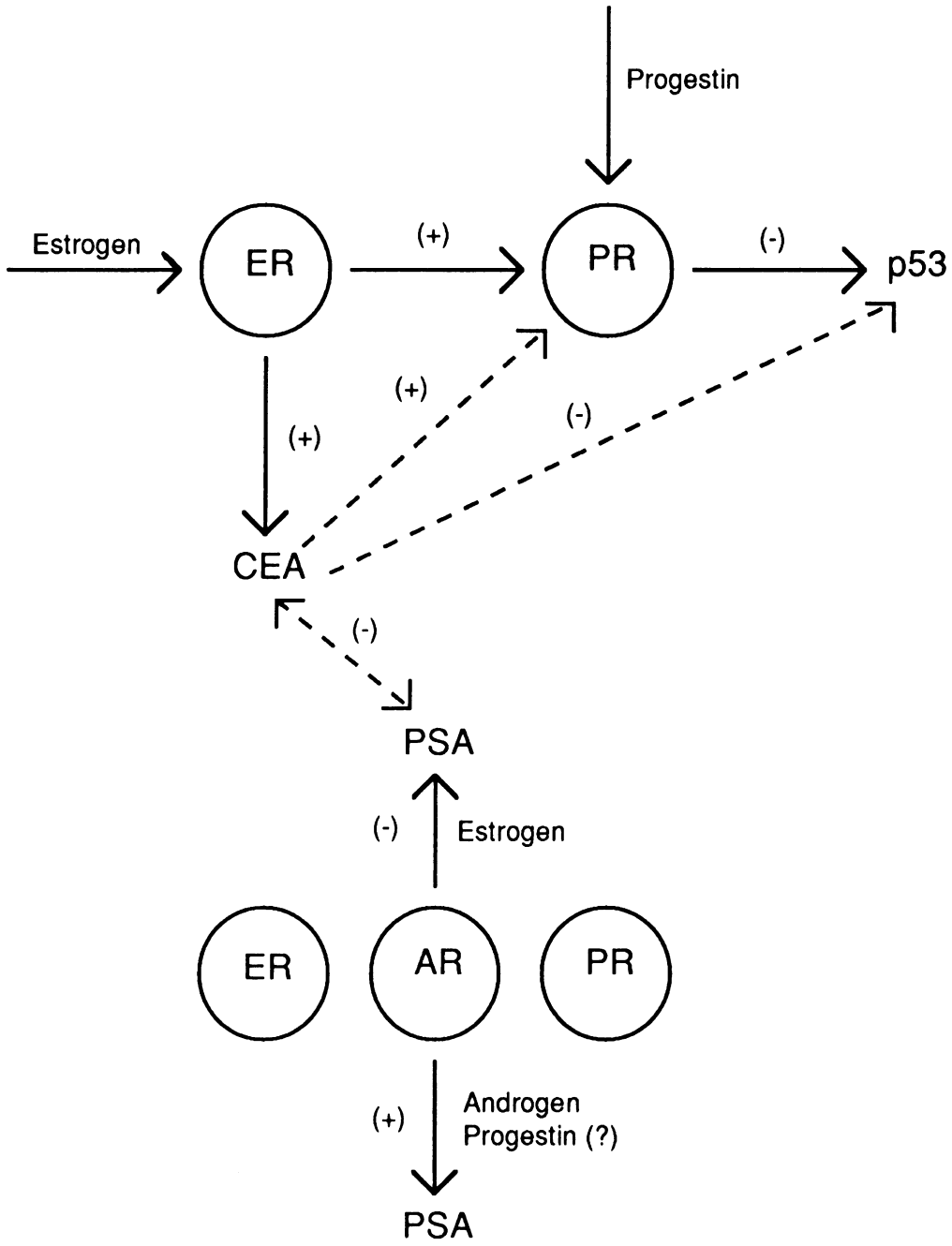

Fig. 6. Model for explaining the relationship between the five studied biochemical parameters in breast tumors. ER, PR, and AR are estrogen, progesterone, and androgen receptors, respectively. (+) on solid arrows denotes stimulation. (−) on solid arrows denotes inhibition. The broken lines indicate indirect positive (+) or negative (−) associations which are the result of the proposed direct relationships between the biochemical parameters (shown by solid arrows). For more discussion see text.

[65], we propose a model which could explain the complex associations between receptors, p53, CEA, and PSA (Fig. 6). In this paper, we have found that the p53 protein is strongly negatively associated with both the ER and PR and that the association becomes stronger as the p53 cutoff level is increased (Table 3). However, the group of tumors which are positive for only one receptor (ER or PR) is not associated with p53 presence or absence at any level of p53 studied (Table 4). We have interpreted this as an indication that the p53 gene is regulated by both the ER and the PR in breast cancer. The negative association between receptors and p53 calls for a repression mechanism likely operating through PR-progestin complexes. It is widely known, as shown in Fig. 6, that the PR is under the control of the ER. Thus, our model is compatible with the finding that p53 is linked to both the ER and the PR.

The CEA gene derepression is likely under the

control of steroid hormone receptors. This is proposed based on the very strong associations between CEA presence and receptors, shown in Table 6. However, it is also evident from the data of Table 7 that tumors positive only for ER are also associated with CEA presence and tumors positive only for PR are not associated with CEA presence. These data support the hypothesis that the CEA gene is regulated by estrogens acting through the estrogen receptors. This view is further strengthened by the findings in Table 6 of much stronger associations between estrogen receptors and CEA in comparison to progesterone receptors and CEA. The observed associations between CEA and progesterone receptors would be expected because ER and PR are known to be associated with each other as shown in Fig. 6 [52]. Likewise, based on the negative associations between p53 and ER and PR, it is expected that CEA and p53 would be negatively associated with each other (confirmed by the data of Table 5).

The PSA gene is under the control of androgens, progestins, and estrogens. Although the mediating receptors in breast cancer cells have not as yet been identified, we have found that in the breast cancer cell line T-47D, androgens and progestins derepress and estrogens repress the PSA gene [66]. These associations are diagrammatically shown in Fig. 6. The weak negative associations between CEA and PSA, identified in Table 8, could be explained by considering that estrogens bound to estrogen receptor can induce CEA production as already proposed, and at the same time block PSA production, the latter shown in a tissue culture system involving T-47D breast tumor cells.

Discussion

The present investigation is based on a large number of tumors which were analyzed for traditional and new candidate prognostic markers using quantitative and highly sensitive immunofluorometric techniques. The quantitative data for the p53 tumor suppressor gene product which, as we have shown, can be obtained reproducibly over long periods of time (Table 2) were analyzed at various cutoff levels as shown in Tables 3–5. This analysis clearly demonstrated the strong negative association between p53 and the ER and PR and established that as the p53 cutoff concentration is increased, the negative association becomes progressively stronger. Such analysis was previously impossible to be performed because of the qualitative nature of immunohistochemical p53 assays. Our findings suggest a close linkage between p53 and receptors and a possible control of the p53 gene by receptors bound to steroid ligands. We were also able to show that CEA-positive tumors are closely associated with ER-positive tumors. This association was found in tumors which were PR-negative but not in tumors which were only PR-positive. Based on this observation, we suggest that the CEA gene is closely linked to the ER and is likely derepressed by ER-estrogen complexes. The positive association between CEA and PR and the negative association between CEA and p53 are likely indirect and originate from the direct relationships between ER and CEA, ER and PR, and PR and p53 (Fig. 6).

We have previously shown that PSA-positive tumors are strongly associated with ER and PR-positive tumors [65] and that PSA production is induced by androgens and inhibited by estrogens [66]. This finding explains why CEA-positive tumors, indicative of the possession of ER-estrogen complexes, are associated with PSA-negative tumors.

Some previous studies found no linear correlation between CEA and receptors [52], in accordance with our data. However, lack of linear correlation does not exclude the presence of a strong association which, in fact, exists, as shown in Table 6. As we have previously shown [65] the steroid hormone receptors are necessary but not sufficient to mediate the events of steroid hormone-receptor complexes. Thus, in many tumors, the receptors may be present, but these are inactive at the level of gene derepression (e.g. CEA or PSA genes) or repression (e.g. p53 gene) in the absence of the steroid hormone ligands. On the other hand, the presence of PSA or CEA in tumors would be closely linked to the presence of the receptors but not necessarily to their absolute concentrations because the magnitude of the receptor-mediated effect would be directly dependent on the availability of ligands.

In breast cancer, estrogens are known to be unfavorable ligands and androgens/progestins are known to be favorable ligands. Therapy that is directed toward blocking the estrogenic effects or shifting the balance in favor of androgens/progestins is currently widely used. In our model, we propose that the p53 presence may be indicative of progestin deficiency in ER and PR-positive tumors and a consequence of receptor absence in ER and PR-negative tumors. We also propose that the presence of CEA in ER-positive tumors is an indicator of estrogenic action in the tumor microenvironment, an unfavourable prognostic sign. The presence of PSA in the tumor is an indicator of the dominance of androgen-progestin action over estrogenic influences.

Breast tumors originate, grow and spread in an environment which involves steroid hormone receptors, various steroid ligands, and gene products regulated by steroid hormone receptor-ligand complexes. In this report we have quantitatively studied three gene products which are currently being evaluated as potential prognostic indicators in breast cancer, namely p53, CEA, and PSA. In this large series of breast tumors we have shown that our quantitative and highly sensitive assays are suitable for routine use. We have further identified previously unrecognized associations between the three tumor markers and receptors and proposed a model which explains the relationships. These associations further help in the understanding of the biology of breast tumors and hopefully could lead to new ways for breast tumor prognosis, diagnosis, and therapy.

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