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Serological and Immunohistochemical Detection of a 65-kDa Oncofetal Protein in Breast Cancer

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Sera from 132 breast cancer patients and 112 healthy female controls were tested with a double-antibody sandwich ELISA using two different monoclonal antibodies against the 65-kDa oncofetal protein, termed p65. Of cancer sera, 90.2% were positive for p65. The average level of p65 was 466.5 ± 243.8 ng/ml (mean ± S.D.) in carcinomas and 37.4 ± 29.5 ng/ml (mean ± S.D.) in controls ($P < 0.0005$). A selected group ($n = 15$) of these 132 patients were needle-biopsied and assessed immunohistochemically using monoclonal antibodies against p65. Nucleocytoplasmic expression was found in 12 patients (80%) using monoclonal antibodies. Expressions of p65 were concordant in 13 (86%) cases between serum and tumour tissues, but did not correlate with tumour DNA ploidy, histological grade or hormone receptors levels. Sera were also tested for CA 15-3 with the average value in cancer serum being 132.4 ± 14.0 U/ml; there was no significant concordance between the two markers. Thus, p65 may be a potential serum and/or immunohistochemical marker for breast carcinoma.

Key words: p65, breast cancer, CA 15-3, ELISA, immunohistochemistry, antibodies
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INTRODUCTION

BREAST CANCER is the most frequent malignancy in adult women [1]. It is thus important to find tumour markers to detect this disease at an early stage (possibly in asymptotic individuals) to identify recurrences early, to monitor response to the various medical, surgical or radiological therapies and to evaluate prognosis [2]. Previously, we found that a 65-kDa oncofetal phosphoprotein, termed p65, specific for transformed cells, appeared in the blood of cancer patients, and tumour-bearing animals [3]. These data suggested that p65 may be expressed by many types of tumour cells. We found this phosphoprotein in both human and rat amniotic fluid and in fetal rats at 15–18 days of gestation, but it has not been detected in the maternal blood. We found that p65 was produced by and released into cell culture medium by transformed cells but not by untransformed cells [4, 5]. Furthermore, this factor is not induced by the cellular proliferation associated with non-neoplastic diseases and conditions and is present in very low levels in the serum or plasma of healthy subjects [3–5]. It has not been detected in the rat during liver regeneration (hyperplasia) in response to partial (2/3) hepatectomy. The p65 was found to be present in premalignant foci observed in the early stages of chemical hepatocarcinogenesis [6]. It was apparent from analysis of the foci that anti-p65 antibodies detected only foci that were tumour-promoter independent [4, 6].

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Recently, we purified p65 to homogeneity from conditioned media, from the rat transplantable hepatocellular carcinoma cell 1682C, and from the human breast carcinoma cell line MCF-7. Lectin-affinity immunoblot analysis using different lectins revealed the presence of carbohydrate residues on the p65 molecule. This protein was found to be phosphorylated *in vivo* on tyrosine, threonine and serine [5]. The purified human and rat p65s showed very high homology and were used to induce immune responses in C57BL/6N mice. Hybridoma cell lines secreting monoclonal antibodies (MAbs) to p65 were established, and a rapid and sensitive, sandwich-type enzyme-linked immunosorbent assay (ELISA), using purified MAbs, was developed to measure the p65 in sera from tumour-bearing rats and from cancer patients with malignant tumours of the breast and prostate. We have found that in all cancer sera tested in a small preliminary study, p65 was markedly elevated [7, 8].

In the study reported here, elevated levels of p65 were detected with MAbs in both the serum and tissues of patients with carcinoma of the breast. The results were compared with another marker, CA 15-3, often used in breast cancer detection, but no significant correlation was observed.

MATERIALS AND METHODS

Tissue and serum samples

For preliminary ELISA studies, a total of 312 sera from healthy females, early and advanced breast cancer patients and benign disease patients, were obtained from the cancer hospital. Blood was collected from 132 patients admitted to the cancer hospital with ductal-type adenocarcinomas of the breast. Their tumours were in the following stages (UICC-AJC 1992): 25 stage 0, 14 stage I, 31 stage IIA, 21 stage IIB, 22 stage IIIA, four stage IIIB and 15 stage IV. The patients' ages ranged from 33 to 88 years, mean 52.6. 15 previously untreated patients from this group were selected for prospective biopsies at the Institute

Curie, Paris, and representative pieces of tumour tissue were embedded in paraffin after brief formalin fixation. The remaining fragments were stored in nitrogen until use for hormone receptor analysis. Sections were stained with hematoxylin and eosin. According to Scarff-Bloom (SB) grading [9], one tumour in this group was grade 1, 10 were grade 2 and four were grade 3. Five biopsies showed non-tumoral glandular tissue, and six showed foci of carcinoma *in situ*. Serum samples were coded and detected in a blinded fashion. At the same time, 112 normal human sera were tested to establish the level of p65 in controls. The normal samples came from healthy blood donors, consisting of 112 women, and their ages ranged from 22 to 55 years with an average of 38. Sixty-eight samples were obtained from patients admitted to the hospital with benign breast diseases; this group of patients comprised 60 cases of fibrocystic breast disease, 3 cases of fibrosclerosis and 5 cases of fibroadenosis (patients' age range 34–67 years). The diagnoses were based on the clinical picture and on the results of histological and radiological diagnosis. Blood was collected in the early morning, then the serum was separated and, after aliquoting, stored at −70°C. All samples were analysed in duplicate in at least three different assays.

Monoclonal antibodies against p65

The p65 antigen was purified from conditioned medium from the transplantable rat hepatocellular carcinoma cell line 1682C as described earlier [5] and from medium from the breast carcinoma cell line MCF-7, using basically the same method [8], with slight modifications such as omitting the molecular sieving column separation and replacing the Rotofor concentration step with trichloroacetic acid precipitation. Fractions containing p65 were separated further by isoelectric focusing on native gels. After the gels were stained with Comassie brilliant blue, the p65 band was cut out with a sharp razor and further purified by electrophoresis on a 12.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS). Next, the proteins were transferred to nitrocellulose membranes by the method of Towbin *et al.* [10]. The protein bands were then stained with Ponceau S, and the p65 band was cut out and dissolved in the smallest possible volume of dimethyl sulfoxide. This antigen solution was then mixed with complete (for first administration) and incomplete (for second and third injections) Freund's adjuvant. C57BL/6N mice for MAbs were immunised by standard procedure [11]. The MAbs were purified on Protein A-Sepharose, and were classified as IgG_1 subclass. Two MAbs, MHB2 and MHF11, which specifically reacted with p65 in the serum of cancer patients but had almost no reaction to normal human serum, were selected for double-antibody sandwich ELISA [8].

Quantitation of p65 *in human sera by double-antibody sandwich ELISA*

MHB2 and MHF11 were used together in double-antibody sandwich ELISA to quantitate p65. Ninety-six-well microtitre plates were coated with 2 μg/ml MHF11 and incubated at room temperature for 3 h. The plates were then blocked with 3% bovine serum albumin (BSA) in 10 mM phosphate buffered saline (PBS) with Tween 20, pH 7.4 (PBST), at room temperature overnight, and then washed three times with PBST. Human serum samples (25 μl) were diluted 1:1 with 3% BSA in PBST and added to plates coated with the first MAb. To construct standard curves, we added known amounts (1–1000 ng/ml) of pure human p65 diluted 1:1 with PBST. The preparation was then loaded onto plates incubated for 3 h at room temperature, and then washed and incubated with 50 μg/ml of diluted (1:500) alkaline phosphatase-conjugated secondary monoclonal antibody, MHB2, prepared according to Voller *et al.* [12]. After final washing, the p-nitrophenyl phosphate substrate was added, and after 30 min of incubation in the dark, the absorbance was read at 405 nm on Dynatech Microplate Reader (Dynatech, Chantilly, New Jersey, U.S.A.). The coefficient of variation (CV) was evaluated on a panel of samples with low, medium and high p65 levels. The intra-assay and inter-assay CVs were: for low levels, 8.2–9.3%, for medium levels, 5.9–7.9%, and for high levels, 7.2–9.3%.

Quantitation of CA 15-3 in human sera

The test for CA 15-3 was performed on 95 cancer serum samples, out of the 132 breast cancer serum samples obtained as described above, using an immunoradiometric assay (IRMA), a solid phase assay based on the sandwich principle. The range of normality was established by taking concentrations under the 95th percentile as normal CA 15-3 values. Samples with less than 20 U/ml were considered negative for CA 15-3.

Immunohistostaining of tumour tissues

The breast tumour tissue samples were stained with avidin–biotin–peroxidase after deparaffinisation, as described by Vector (Burlingame, California, U.S.A.). For the second-phase and third-phase reagents we used the Vectastain ABC kit (Vector), containing biotinylated anti-rabbit or anti-mouse immunoglobulins and reagents that form avidin-biotin complexes. The tissue sections were washed with PBS, incubated with diluted 1:100 MAb, MHF11, for 30 min, washed again with PBS, incubated with the second-phase and third-phase reagent for 30 min, and finally washed with PBS. The sections were then stained for 5 min with 0.6 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St Louis, Missouri, U.S.A.) in PBS containing 0.01% hydrogen peroxide. Endogenous avidin–biotin activity was blocked by the method described by Wood and Wranke [13]. The tissue sections were incubated with avidin solution for 5 min, washed, and incubated with biotin solution for 5 min before immunostaining. In the group of 15 biopsied patients, 6 have been diagnosed as having carcinoma *in situ*. Immunoreactivity was graded as 1 for weak and selective staining of less than 25% of the tumour area; 2 for strong staining of 25–75% of the tumour area; and 3 for very strong staining of more than 75% of the tumour area.

Biochemical assays of oestrogen and progesterone receptor levels

Radioligand assays were performed on 13 of the 15 biopsy samples from the group of 15 drill-biopsied patients. Positivity for oestrogen receptor (ER) and progesterone receptor (PR) was defined as more than 500 fmol/mg DNA [14].

DNA flow cytometry

Fifteen tumours from the group biopsied as described above were analysed by DNA flow cytometry. The collection and storage of cells for the flow cytometric analysis has been described elsewhere [15]. Suspensions were stained using a previously reported one-step protocol [16]. Two tumours were diploid and 13 were aneuploid.

Statistical methods

Statistical analysis was performed at the University of Texas, M.D. Anderson Cancer Center, Department of Biomathematics for one-way analysis of variance, frequency distribution and linear regression [17].

RESULTS

Measurements of circulating p65 and CA 15-3 antigens in breast cancer patient sera

A linear standard curve, with a correlation coefficient of 0.97, was determined by using pure human p65 in concentrations ranging from 0 to 1000 ng/ml, in order to quantitate the level of p65 in unknown serum samples and also to determine the reproducibility of the method. The coded sera from 132 cancer patients, diagnosed previously with breast cancer, were then analysed in a blinded fashion. As shown in Figure 1, 119 of 132 serum samples from breast cancer patients were positive for p65 (90.2%), as indicated by p65 levels that were at least two standard deviations above the mean level in normal human sera. The mean value was 466.5 ± 243.8 ng/ml, and the range was 135.2 to 958.9 ng/ml. The mean serum level for p65 in 112 normal healthy controls was 37.4 ± 29.5 ng/ml. At the same time in the group of patients with benign breast diseases, i.e. patients admitted to the hospital for non-cancer-related causes, p65 levels were slightly elevated above the mean, with the average p65 value of 74.0 ± 41.2 ng/ml. Of the patients in this group, 80% were negative for p65. Thus, the mean value for cancer serum was significantly ($P < 0.0005$) higher than the mean p65 value for normal human sera or benign breast diseases sera. An increased frequency of pathological results in the breast cancer population was found by the χ^2 test ($\chi^2 = 137.1$; $P < 0.0005$). A significant difference among groups was documented by means of the analysis of variance (ANOVA, one way; $F = 32.1$, $P < 0.01$). Serum p65 levels correlated with pathological stage of the disease, as is shown in Figure 2 which depicts the relationship of serum p65 and clinical cancer stage for 132 breast cancer patients. The shaded area represents the cut-off level used in our study, where the mean plus two S.D. (96 ng/ml) were used as the upper limit of normal p65 expression. Elevation of p65 was not obvious in patients with pathological stages 0 and 1, but in stages IIA, IIB, IIIB and IV showed a marked increase above the cut-off point as shown in Figure 2. In the group of cancer patients selected for biopsies, p65 levels were significantly ($P < 0.005$) above the mean, with values ranging from 135.0 to 488.8 ng/ml. The mean value of serum p65 for this group of patients was 220.0 ± 111.2 ng/ml. A normal frequency distribution pattern for p65 was observed in the studied group of breast cancer patients (data not shown).

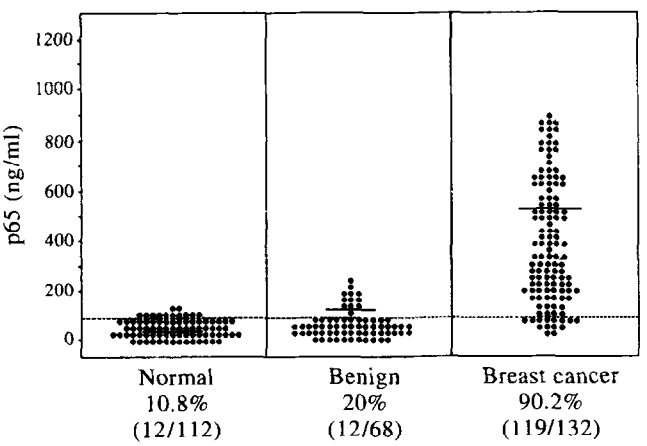

Figure 1. ELISA serum p65 values in breast carcinoma, benign breast disease and control subjects. Dotted and continuous lines represent the cut-off level and p65 positivity, respectively. % of positivity is also shown for all three groups.

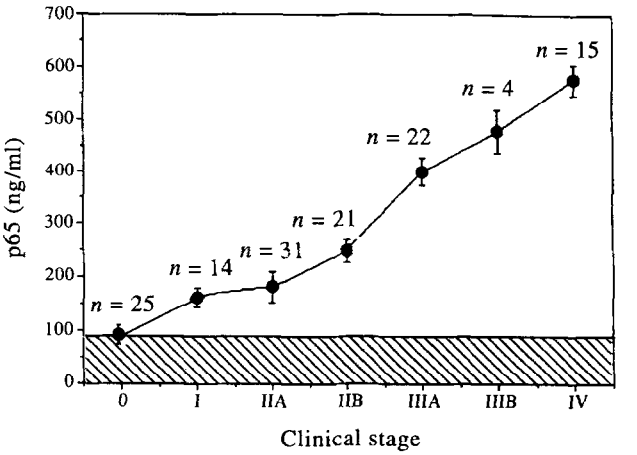

Figure 2. Relationship of serum p65 and clinical stage for 132 breast cancer patients. The mean plus two standard deviations (96 ng/ml) was used as the upper limit of normal p65 expression. The samples under the cut-off line (shaded area) were considered negative and those above positive.

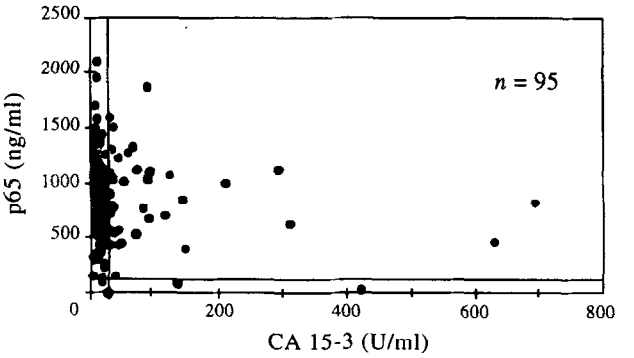

Figure 3. Lack of correlation between p65 and CA 15-3 values in breast cancer sera.

Figure 3 reports the lack of correlation between p65 and CA 15-3 levels in the group of 95 breast cancer patients. The serum levels of CA 15-3 antigen in the cancer patients were determined by IRMA and ranged from 29.0 to 702.0 U/ml, with a mean of 132.4 ± 14.0 U/ml. Only 45 of 95 patients (47.3%) had CA 15-3 levels above the accepted normal range of 20 U/ml, and only 34 patients (36%) had both markers present in their blood, while 81 of the same group of patients (85.3%) showed an elevated p65 level. Table 1 shows the specificity, sensitivity and predictive value for marker elevation for p65 in comparison with CA 15-3. As can be seen from Table 2, the incidence of elevated levels of p65 in early and late stage of breast carcinoma development was 90.2 and 90.0%, respectively; the incidence of elevated CA 15-3 was 45.8 and 80%, respectively. A significantly higher percent-

Table 1. Performance indices p65 and CA 15-3 markers in breast cancer screening

	p65	CA 15-3
Sensitivity (%)	90.2	47.3
Specificity (%)	88.5	84.3
Prognostic predictive value for marker elevation (%)	88.2	64.5

Table 2. Elevated serum levels of p65 and CA 15-3 in patients with breast carcinoma

	Disease status					
	Early		Late		Early and late	
	Elevated/total	%	Elevated/total	%	Elevated/total	%
p65 > 100 ng/ml	83/92	90.2	36/40	90.0	119/132	90.2
CA 15-3 > 20 U/ml	41/90	45.6	4/5	80.0	45/95	47.4
Both markers	30/90	33.3	4/5	80.0	34/95	35.8
At least one marker	81/90	90.0	4/5	80.0	85/95	89.5

age of patients with early active disease had elevated levels of p65 in comparison with CA 15-3. However, in all advanced cases both markers were highly elevated.

The clinical and biological profile of the biopsied patients is shown in Table 3. In addition to p65 and CA 15-3 serum levels, we determined ER and PR distribution as well as Scarff-Bloom grade and p65 levels in the tumour tissue. Eight tumours were positive for ER (1299–18400 fmol/mg DNA), and four were positive for PR (725–5600 fmol/mg DNA). Four tumours were both ER- and PR-positive, four were ER-positive and PR-negative, and five were ER- and PR-negative. Continuously increasing serum p65 values in carcinoma patients may indicate tumour progression. There is also a slightly positive correlation ($r^2 = 0.59$) between immunohistochemistry staining and the ELISA results in sera of 15 patients selected for comparisons. Eleven of 15 tumours (73%) were stained positively by anti-p65 MAb (Table 3). As indicated in Table 4, normal glandular tissues which were stained with anti-p65 MAb did not show reactivity. In addition, four of six carcinomas *in situ* stained with anti-p65 MAb (Table 4). Both the cytoplasms and nuclei of the tumour tissue reacted. Cytoplasmic immunostaining was distinct and homogenous in all cells using monoclonal antibodies. Nuclear immunostaining was stronger than cytoplasmic staining with an affinity for chromatin granulations (Figure 4). MAb stained epithelial elements strongly and there was almost no background staining. Staining intensity is presented in Table 4. While serum and immunohistochemical expression of p65 correlated, there was no specific correlation between p65, hormone receptor levels, ploidy, histological grading or presence of CA 15-3.

Table 4. Immunostaining of normal tissue, carcinoma in situ *and adenocarcinoma in a selected group of breast cancer patients, using monoclonal anti-p65 antibody MB2*

Patient case number	Normal tissue	Carcinoma *in situ*	Adenocarcinoma
1	ND	+	+
5	ND	++	+++
7	+/−	ND	++
10	−	ND	−
12	ND	−	−
13	−	+	++
14	+/−	+/−	+

ND, not determined; +, positive; ++, moderately positive; +++, strongly positive; +/−, equivocal; −, negative.

Table 3. Characteristics of selected biopsied patients

Patient	Stage	Age (years)	Ploidy	Tissue* SB‡	Tissue* p65	Serum p65† (ng/ml)	ER‡	PR‡	Serum CA 15-3§ (U/ml)
1	IIA	44	A	3	1	164	90	220	20
2	IIB	60	A	2	1	164	1299	179	—
3	IIIB	61	A	3	2	288	50	130	153
4	IIA	62	A	2	3	360	130	40	—
5	IA	60	A	2	1	156	4010	250	10
6	IIB	57	D	2	3	344	—	—	14
7	IIA	43	D	2	2	220	2060	1400	27
8	IIA	61	A	1	2	228	1580	920	21
9	IA	70	D	2	3	420	18400	5600	—
10	IIA	41	A	2	2	236	1840	210	31
11	IIB	61	A	3	3	488	170	400	23
12	IIA	71	A	2	3	360	—	—	—
13	IA	70	D	2	2	276	4600	100	—
14	IIA	44	A	2	3	280	4430	725	31
15	IIA	60	A	3	1	148	480	180	—

SB, Scarff-Bloom grade. *p65 immunoreactivity grade determined by tumour tissue staining; †p65 values determined in serum by ELISA; ‡fmol/mgDNA oestrogen (ER) and progesterone (PR); §Serum CA 15-3 values determined by IRMA. A, aneuploid; D, diploid; —, not determined.

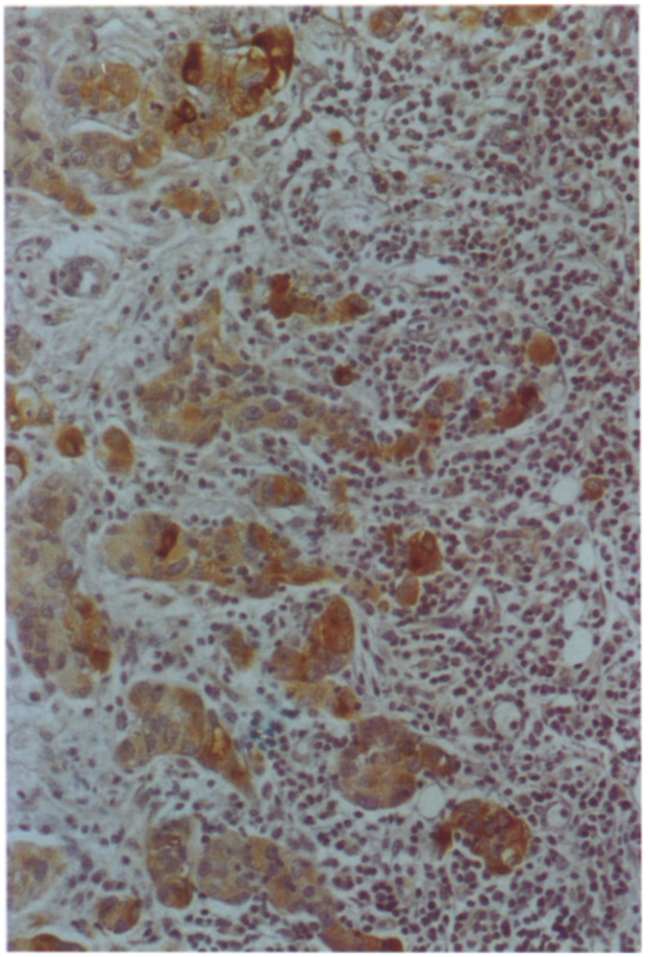

Figure 4. Immunohistochemical staining of paraffin-embedded breast carcinoma (case 14 from Table 2) with monoclonal anti-p65 antibodies. Note strong cytoplasmic and selective nuclear positivity of tumour cells. Avidin–biotin–peroxidase. Magnification × 100.

DISCUSSION

Malignant cells produce substances that are not present in normal cells, but finding and identifying clear-cut markers is very difficult. Many markers have been discovered and characterised as useful for cancer detection and for monitoring cancer therapy, but very often they are present at very low levels in normal cells and in some non-malignant conditions as well [17]. For a marker to have maximal usefulness, it must be expressed by all or most cancerous or transformed cells [18]. The best examples of tumour markers used in the clinic today are carcinoembryonic antigen (CEA), α-fetoprotein, tissue polypeptide antigen, pancreatic oncofetal antigen, and others like CA 19-9 or CA 12-5 [19]. Serum CEA levels have been used to monitor tumour growth and metastasis [20], but CEA has been criticised because of its low sensitivity and low specificity. Other markers such as HFMGI, HFMG2 and 115D8 have also been investigated [21]. In 1987, CA 15-3 was introduced as a helpful tool in diagnosis of breast cancer [22]. CEA and CA 15-3 are not related to well-known prognostic parameters such as ER and PR levels and histological grading [21]. In general, the serum markers used in breast cancer detection have low sensitivity and specificity, but can be used to monitor tumour relapse.

Our earlier experiments indicated that both MAbs and PAbs against rat p65 could detect both early and late neoplastic changes during chemically-induced rat hepatocarcinogenesis [6]. In the study reported here, we observed immunohistochemically-specific, apparently progressive expression of p65 from no expression in normal human breast tissue, through weak carcinoma *in situ* expression to strong expression in invasive carcinoma.

We also successfully developed a double-antibody sandwich ELISA and immunohistochemical analysis to measure the expression of p65 in serum and tissue from breast cancer patients. Interestingly, our earlier studies have shown that p65 immunohistochemical expression increased with tumour progression [8].

The utility of a new tumour marker test depends on its specificity and sensitivity. The specificity of the marker for identifying malignancy is dependent on the degree by which it separates normal, healthy individuals from cancer patients. We found that the concentrations of circulating p65 in sera of patients with breast carcinoma were significantly higher than those found in the sera of normal subjects ($P < 0.0005$). More than 90% of sera from subjects with breast cancers were positive for p65, a higher percentage than for CA 15-3 (47%). However, there was some overlap with the normal range, which indicates that the p65 test is not absolutely specific. In this preliminary study, we found symmetrical frequency distribution of p65 in the breast cancer group. The results, however, are based on a relatively low number of cases and will require further work on an increased sample pool to prove the assay to be more sensitive and accurate. Serial sample determinations are currently being performed in our laboratory, in order to establish how p65 fluctuates with the tumour burden changes, resulting from disease progression or therapy intervention. Then changes in serum p65 levels could be used to monitor breast cancer progression and regression. Using the avidin-biotin-peroxidase technique on paraffin sections, we were able to show specific staining in both nuclei and cytoplasm. Presumably, p65 is distributed in this fashion because it is a shed antigen and is present in extracellular space and on the surface of cell membranes during its release from the production size to the peripheral blood. This result is similar to that of rat liver hepatocarcinogenesis experiments [6]. Because of p65's immunolocalisation in nuclear membranes and its phosphorylation on tyrosine [5], we assume that p65 may facilitate the regulation of tumour genes. We are fully aware that none of the markers available today are by all means specific and we need to learn more about the performance of the p65 marker. Our p65 data obtained from breast cancer patients offer additional information to the clinician, especially when used in combination with other markers like CA 15-3 and seem to indicate that this marker can be used for diagnosis relatively early in the development of breast cancer. This early detection may improve care and therapy of the individual patient, allowing the clinician to start intervention earlier and at the stage where recurrent process is not so obvious.

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c-*myc* mRNA Overexpression is Associated with Lymph Node Metastasis in Colorectal Cancer

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To elucidate the relationship between oncogene overexpression and malignant potential of colorectal cancer, the expression of three genes, c-*myc*, c-*fos* and c-Ki-*ras* was examined. Northern blot hybridisation was performed on fresh tissues excised from 35 patients with primary colorectal cancer. Overexpression of c-*myc* mRNA was evident in 25 of the patients (71%). Tumours with c-*myc* overexpression had a significantly higher frequency of lymph node metastasis than did those without (68 versus 20%, $P < 0.05$). The survival rate tended to be poorer ($P = 0.06$) in patients with c-*myc* overexpression (79% for 3 years) than in those without it (100%). We found no significant relationship between overexpression of the other two genes and pathological features. These findings suggest that c-*myc* overexpression is one parameter which can be used to determine the malignant potential of colorectal cancer.

Key words: colorectal cancer, c-*myc* mRNA overexpression, northern blot analysis, clinico-pathological findings, prognosis

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INTRODUCTION

PROGNOSTIC FACTORS in case of surgical resection of colorectal cancer are the presence or absence of metastasis [1, 2], depth of tumour invasion and venous and lymphatic invasion [3]. Recent molecular biological studies suggested that estimation of oncogene overexpression may be useful in predicting the prognosis [4, 5].

Certain cellular oncogenes seem to be associated with cellular proliferation, carcinogenesis and tumour progression [6]. Extensive studies on the expression of the oncogenes were conducted