Pergamon

European Journal of Cancer Vol. 32A, No. 7, pp. 1148–1155, 1996

Printed in Great Britain
0959–8049/96 $15.00+0.00

PII: S0959-8049(96)00037-8

Original Paper

Immunohistochemical Evaluation of Multiple Biological Markers in Ductal Carcinoma *In Situ* of the Breast

T. Perin,[1] V. Canzonieri,[1] S. Massarut,[2] E. Bidoli,[3] C. Rossi,[2] M. Roncadin[4] and A. Carbone[1]

[1]Division of Pathology, [2]Division of Surgical Oncology, [3]Division of Epidemiology, and [4]Division of Radiotherapy, Centro di Riferimento Oncologico di Aviano, Istituto Nazionale di Ricovero e Cura a Carattere Scientifico, Italy

In order to obtain prognostic clinicopathological information, 49 cases of pure ductal carcinoma *in situ* of the breast (DCIS), were evaluated for the immunohistochemical expression of potential predictor markers including c-erbB-2 oncogene product, p53 protein, oestrogen (ER) and progesterone (PR) receptors, oestrogen-regulated proteins pS2 and cathepsin-D (cath-D), CD44 protein and 67-kDa laminin receptor (MLuC5). Immunohistochemical findings were compared with conventional pathological parameters, clinical findings, and the clinical outcome of the patients. When markers were matched to each other, statistical analyses provided a significant positive correlation between c-erbB-2 overexpression and p53 positivity ($P < 0.01$) and between ER and PR ($P < 0.01$), ER, PR and pS2 ($P < 0.01$), pS2 and MLuC5 ($P < 0.05$). Significant negative correlations between c-erbB-2 overexpression and ER ($P < 0.05$), PR ($P < 0.01$) and pS2 ($P < 0.01$) positivity was also observed. Data on the relationship between marker status and pathological findings revealed a significant positive trend between c-erbB-2, p53, and increased grade values ($P < 0.05$) and opposite results with PR receptor expression ($P < 0.01$). c-erbB-2 overexpression was further significantly associated with comedotype carcinoma ($P < 0.05$) and distribution of disease in confluent neoplastic ducts ($P < 0.01$). Although no statistically significant correlation among biological markers expression, clinical findings and outcome was demonstrated, overall this study indicates that tumour cells from a subset of DCIS, which includes comedotype carcinoma, express significantly unfavourable prognostic factors.

Key words: c-erbB-2, p53, ER, PR, pS2, cath-D, CD44, MLuC5, ductal carcinoma *in situ* of the breast
Eur J Cancer, Vol. 32A, No. 7, pp. 1148–1155, 1996

INTRODUCTION

INTRADUCTAL CARCINOMAS of the breast have been increasingly diagnosed in the last 20 years. Until 1970, they were a relatively rare condition, accounting for 2% or less of overall breast cancer. The present figures of 15–20% mainly reflect both the high sensitivity and the wide diffusion of diagnostic procedures [1, 2].

Nevertheless, uncertainty persists on the correct therapeutic approach to this pathological condition, mainly because of the poor definition of biological and clinical implications of a number of pathological characteristics [3, 4]. Only comedotype carcinomas, usually showing some dismal morphological features, have been confirmed to have higher recurrence and progression rates, as compared to the other histotypes [5].

However, biological, radiological and clinical heterogeneity of ductal carcinoma *in situ* (DCIS) of the breast should indicate that a pathological, prognostically oriented, classification would be highly desirable to obtain consistent therapeutic guidelines and to stratify patients' groups potentially receiving different treatment schedules. Recently, some proposed classifications [6] have attempted to achieve this goal, but their validation is under scrutiny and will probably depend on their reproducibility and diffusion among pathologists and clinicians.

Interestingly, a number of biological markers have been proposed as prognostic indicators in DCIS, such as c-erbB-2 [7–9], p53 [10, 11] and oestrogen and progesterone receptors (ERs and PRs) [12, 13]. However, controversy exists regard-

Correspondence to A. Carbone
Received 30 Aug. 1995; revised 4 Dec. 1995; accepted 11 Jan. 1996.

ing the value of some of these prognostic factors, their interrelation [14–16] and their advantages over better known prognostic characteristics such as histological type, cytological grading, extension and distribution of disease.

The need for more information led us to perform a clinicopathological and immunohistochemical study on a well characterised series of DCIS, taking into consideration: (1) the tumoral expression of a panel of potential predictors including oncogenes, hormonal receptors and hormone-dependent proteins, (2) their correlation with morphological parameters and (3) the outcome of patients.

PATIENTS AND METHODS

From January 1984 to December 1992, 49 case of pure DCIS were diagnosed at the C.R.O. Aviano (Italy). The median age of the patients was 52.5 years (range 38–84 years). At presentation, 24 patients (49.0%) had clinical signs of disease, while in the remaining 25 patients (51.0%) the diagnosis was based on mammographic findings. 13 patients (26.5%) underwent mastectomy and 36 (73.5%) conservative surgery. Axillary node dissection was performed in 38 patients (77.6%). Of the 36 patients treated with conservative surgery, 30 (83.3%) received radiotherapy to the remaining breast.

To determine the gross pathological features of the tumours, all the original pathology reports were reviewed. All available histological slides from each mammary specimen (mean 25 paraffin blocks of tissue, range 6–82) were "blindly" revised by two of us without knowledge of the clinical outcome. A mean of 10 tumour documenting slides per case (range 1–30) were found. The most representative section from each case was selected for immunohistochemistry.

The following pathological parameters were evaluated: extension and distribution of disease, histological subtype and cytological grade of malignancy. When present, ancillary histological features such as microcalcifications, lymphoplasmacytic infiltrate around involved ducts and concentric periductal fibrosis (to characterise a possible host reaction) were also recorded. A separate scoring for each parameter was used. In the case of lymph nodes dissection, the corresponding slides were also reviewed.

The extension and distribution of DCIS were estimated considering three different patterns of growth: scattered neoplastic ducts, confluent neoplastic ducts and nodular area. Scattered tumours were defined when one or few neoplastic ducts were detected in a wide intervening area of mammary tissue; confluent tumours accounted for a lesion having a segmental distribution and, finally, nodular area was defined when the tumour had macroscopic evidence. The primary histological architectural pattern of DCIS was classified according to the established subtypes: comedo, solid, cribriform, micropapillary, papillary, clinging and cystic carcinoma. When the tumour was composed of more than one subtype of DCIS, all architectural patterns were recorded and the tumour was classified in a mixed histological category [17]. For statistical analyses, the tumours were subdivided into comedotype and non-comedotype. The cytological grade of malignancy was evaluated as grade 1, grade 2 and grade 3, depending on the increased mitosis count and degree of nuclear polymorphism; the prominence of nucleoli and the lack of cellular polarisation also characterised the highest grade group [6].

Immunohistochemical study was performed by using a panel of monoclonal (MAb) and polyclonal (PAb) antibodies recognising, respectively, the ER (MAb ER1D5; Immunotech S.A., Marseille, France), the PR (MAb 10A9; Immunotech S.A.), the oestrogen-regulated proteins pS2 (MAb His-pS2-AB1, CIS bio international, Gif-sur-Yvette, France) and cathepsin-D (cath-D; MAb C5, Novocastra Laboratories Ltd, Newcastle upon Tyne, U.K.), the internal domain of c-erbB-2 oncogene product (MAb CB11, Novocastra Laboratories Ltd), the wild and mutant forms of human p53 protein (MAb DO-7; Novocastra Laboratories Ltd), the 67-kD laminin receptor (MAb MLuC5, kindly provided by Dr Sylvie Ménard, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy) and the CD44 protein (PAb; kindly provided by Prof. J. Thomas August, The John Hopkins University School of Medicine, Baltimore, Maryland, U.S.A.).

Immunohistochemistry was carried out in Bouin-fixed, paraffin-embedded tissue sections by using the avidin–biotin peroxidase complex (ABC) method [18] (ABC-Elite kit, Vector, Burlingame, California, U.S.A.). For ER1D5, 10A9, DO-7 and CD44 antibodies, the sections were pretreated in a microwave oven (Jet 900 W, Philips) twice for 5 min at 650 W in citrate buffer (pH 6) before immunostaining. Negative controls, which were invariably negative, consisted of omission of the primary antibody and substitution with phosphate-buffered saline.

Immunohistochemical evaluation was independently performed by two of us with more than 90% accordance. In case of discordant results, agreement was obtained after discussion and by consulting a referring pathologist.

Positivity scoring of immunohistochemistry for c-erbB-2, p53, ER and PR was established according to previously reported criteria [7, 10, 14–16, 19]: c-erbB-2 was considered positive if membrane staining was seen, even in a few cells [7, 15, 16, 19]; p53 was positive if 5% or more cells showed nuclear staining [10, 15]; and ER and PR were positive when any nuclear staining was identified even focally [14–16]. Positivity for pS2, CD44, cath-D and MLuC5 was arbitrarily established when immunostained cells were 10% or more. Specifically, pS2 and cath-D showed cytoplasmic positivity, whereas CD44 and MLuc5 showed membrane positivity. Positive controls for c-erbB-2 and p53 were represented by samples of tumours (invasive breast carcinoma and Burkitt's lymphoma, respectively) previously ascertained as positive for these markers. Conversely, ER and PR were classified as negative when no positive nuclei could be identified; furthermore, it was required that ER and PR staining be present in adjacent normal breast tissue before accepting a tumour as negative. Both normal lymphocytes and samples of colonic neoplasms were used as positive controls for CD44. Similarly, normal hystiocytes were the inner positive controls for cath-D. Finally, normal vasculature represented the positive inner controls for MLuc5.

Since the immunohistochemical variables under study were not normally distributed, non-parametric statistics were chosen. In particular the Mann–Whitney test [20] was used to compare the distribution of c-erbB-2, p53, ER, PR, CD44, cath-D, pS2 and MLuC5 [7, 14, 15], with the main histological features. The following analyses were made: comedotype versus non-comedotype carcinoma, microcalcifications (no versus yes) and distribution of disease, i.e. confluent neoplastic ducts versus scattered neoplastic ducts, nodular area versus scattered neoplastic ducts and nodular area versus confluent neoplastic ducts, microdimensions (<2 cm versus $\geqslant 2$ cm) and cytological grading (G1, G2 and G3).

Spearman's rank correlation [20] was used to study any

possible correlation between immunohistological variables. The chi-squared test [20] was used to test the association between qualitative parameters. Results were considered statistically significant at $P < 0.05$.

Disease-free survival rates between recurrence and histotype and between recurrence and biological marker expression were estimated by the Kaplan–Meier method [21], and the statistical significance between curves was determined by the log-rank test [22].

RESULTS

Clinicopathological findings

None of the patients who underwent mastectomy recurred either locally or distantly; no lymph node metastases were found in any of the 38 patients who underwent axillary node dissection. Out of 36 patients treated with conservative surgery, with or without radiotherapy, 8 experienced local recurrences (22.2%) in a median follow-up of 53 months (range 18–107 months). Recurrences occurred in 2 patients (33.3%) who had not received radiotherapy and in 6 (20.0%) treated with radiation. 4 recurrences were DCIS and 4 were ductal infiltrating carcinomas. The time interval between surgery and evidence of recurrences was 18, 30, 55 and 63 months for DCIS and 19, 54, 60 and 85 months for ductal infiltrating carcinomas, respectively.

Table 1. Pathological findings in 49 intraductal breast carcinomas

	Conservative surgical treatment (36 cases)	Radical mastectomy (13 cases)
Tumour size		
<2 cm	23	6
2–5 cm	9	5
>5 cm	0	0
Unknown	4	2
Histological subtype		
Comedo*	23	7
Cribriform	2	1
Solid	1	1
Papillary	3	0
Micropapillary	6	3
Clinging	0	0
Cystic	1	1
Extension and distribution of disease		
Scattered neoplastic ducts	13	6
Confluent neoplastic ducts	13	3
Nodular area	10	4
Nuclear grade		
G1	8	3
G2	19	6
G3	9	4
Lymphoid infiltrate		
Yes	9	6
No	27	7
Periductal fibrosis		
Yes	12	6
No	24	7
Microcalcifications		
Yes	20	6
No	16	7

*Comedocarcinoma subset includes 16 cases in which the tumour was composed of more than one histotype. The comedotype was consistently detected in these mixed cases but it was dominant only in 9 of the 16 cases.

Figure 1. Strong membrane immunostaining for c-erbB-2 is present in most neoplastic cells (magnification ×400; ABC method; haematoxylin counterstain).

The pathological findings detected in the 49 primary tumours are detailed in Table 1. For statistical analyses, the data were summarised as follows: growth pattern [scattered: $n = 19$ (38.8%); confluent: $n = 16$ (32.6%); nodular area: $n = 14$ (28.6%)]; histological type [comedocarcinoma: $n = 30$ (61.2%); others: $n = 19$ (38.8%)]; cytological grading [G1: $n = 11$ (22.4%); G2: $n = 25$ (51.0%); G3: $n = 13$ (26.5%)]; microcalcifications [yes: $n = 26$ (53.1%); no: $n = 23$ (46.9%)] and host reaction [no reaction: $n = 23$ (46.9%); fibrosis: $n = 11$ (22.4%)], [lymphocyte infiltrate: $n = 8$ (16.3%); both: $n = 7$ (14.3%)]. Statistically significant associations between comedotype carcinoma and high cytological grading ($P < 0.01$), comedotype carcinoma and confluent growth pattern ($P = 0.02$) and confluent growth pattern and microcalcifications ($P < 0.01$) were demonstrated. Distributions between pathological findings and recurrence rate gave the following results: 7 recurrences were found in 23 patients with comedocarcinoma (30.4%) and 1 recurrence in 13 patients with other histologic subtypes (7.7%) ($P = 0.08$), 2 recurrences were found in 13 patients with distribution of disease in scattered neoplastic ducts (15.4%), 4 recurrences in 13 patients with distribution of disease in confluent neoplastic ducts (30.8%)

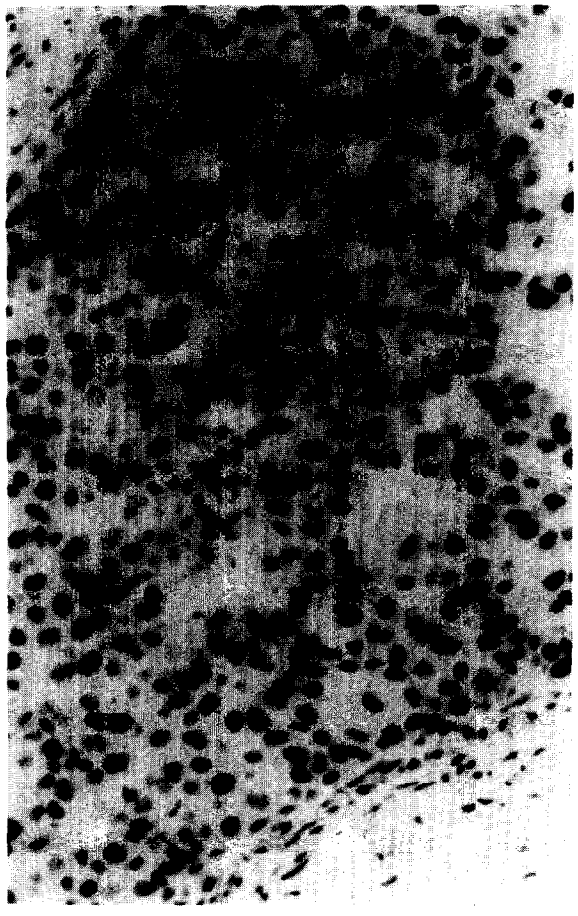

Figure 2. A large number of carcinoma cells exhibit nuclear staining for p53 (magnification ×320; ABC method; haematoxylin counterstain).

Figure 3. Oestrogen receptor staining is nuclear in most neoplastic cells. Some normal small ducts are contiguous to neoplastic ducts (magnification ×320; ABC method; haematoxylin counterstain).

and 2 recurrences in 10 patients with a nodular neoplastic area (20.0%). In respect of nuclear grade, 6 recurrences were found in the 19 patients with grade 2 (31.6%) and 2 recurrence in the 9 patients with grade 3 (22.2%). No recurrence was found in the 8 patients with grade 1.

Immunohistochemical findings

As to the immunohistochemical results, we compared the mean expression of each biological marker to that of the other markers, to the pathological findings as well as to the clinical findings and the outcome.

Firstly, c-erb-B2 and p53 oncoprotein expressions were identified in 55.1% and in 61.2% of the cases, respectively (Figures 1 and 2); 54.2% of the tumours were ER positive, 54.2% were PR positive (Figures 3 and 4) and 62.5% were positive for pS2 (Figure 5).

The MLuC5 antibody was positive in 81.6% of the cases (Figure 6), while CD44 and cath-D were positive, respectively, in 83.0 and 98.0% (Figures 7 and 8) of the cases.

A statistically significant positive correlation was demonstrated between overexpression of c-erbB-2 and p53 immunopositivity ($P < 0.01$) and significant negative correlations were observed between c-erbB-2 overexpression and ER ($P < 0.05$), PR ($P < 0.01$) and pS2 ($P < 0.01$) immunopositivities. A significant positive correlation was also demonstrated between ER and PR ($P < 0.01$) and among ER, PR and pS2 ($P < 0.01$), as well as between pS2 and MLuC5 ($P < 0.05$) (Table 2).

Data on the relationship between biological markers and pathological findings revealed a significant positive association among c-erbB-2, p53 and increased *G* values ($P < 0.05$) and opposite results with PR expression ($P < 0.01$) (Table 3). Moreover, a significant association between c-erbB-2 and comedotype ($P < 0.05$), was found.

As to the distribution of disease, the only significant association concerned c-erbB-2 and confluent neoplastic ducts ($P < 0.01$). Other figures regarding microdimensions and PR and pS2 expression as well as microcalcifications and MLuC5 positivity account for isolated significant results ($P < 0.01$ and $P < 0.05$ for PR and pS2 versus progressively increased microdimensions, and $P < 0.05$ for MLuC5 versus microcalcifications).

Finally, no significant correlation among these biological markers, clinical findings and recurrence rate was demonstrated. However, we observed that the comedocarcinoma was the most frequent histotype encountered in the recurrent tumours, when compared with the other cumulated subtypes (30.4% versus 7.6%) ($P = 0.08$).

DISCUSSION

The present study on 49 DCISs of the breast was aimed at evaluating the expression of selected biological markers, such as c-erbB-2, p53, ER, PR, pS2, CD44, cath-D and MLuC5, and their relationship with some clinicopathological features.

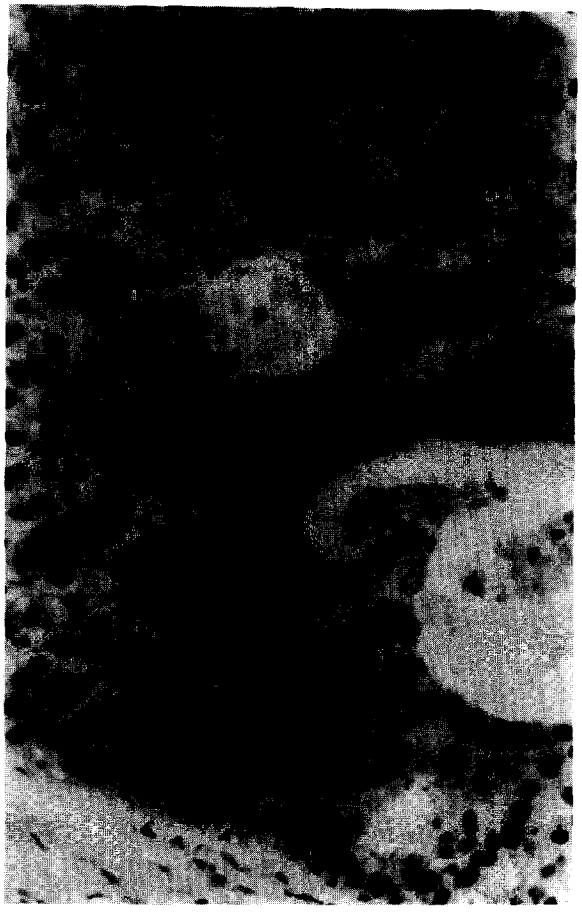

Figure 4. Progesterone receptor staining is shown in most nuclei of neoplastic cells (magnification ×400; ABC method; haematoxylin counterstain).

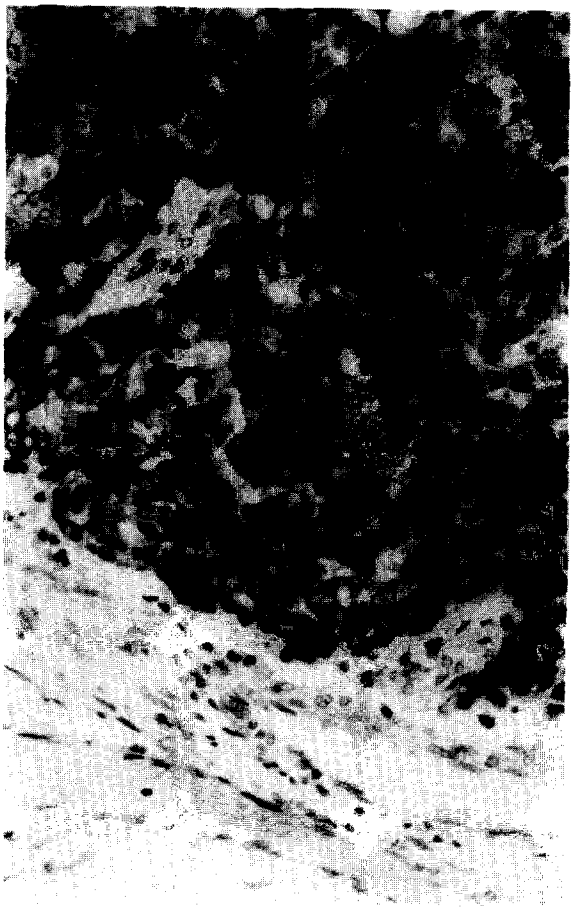

Figure 5. Strong immunopositivity is evident in neoplastic cells with pS2 antibody (magnification ×320; ABC method; haematoxylin counterstain).

As to c-erbB-2 expression, our results are in accordance with previous reported data showing a frequency ranging from 48 to 61% of the DCIS cases [15, 16]. Amplified and overexpressed c-erbB-2 has already been reported in invasive breast cancers. The association between amplification of the *c-erbB-2* oncogene (also called *Neu*) and poor prognosis in human infiltrating breast cancer was first reported by Slamon and colleagues in 1987 [23]. At present, it is well known that its product, the overexpressed Her-2/neu protein, is associated with a more aggressive disease, being an independent predictor of poor prognosis in several subsets of patients.

Concordantly, overexpression of Her-2/neu protein in DCIS indicates a most rapid growth in comedocarcinoma type [24] as well as in large cell type carcinoma and greater lesions.

This is in accordance with the aggressive potential of the DCIS comedo variant based on its histomorphology and on clinical follow-up studies [5]. As shown in the present study, the high degree of correlation of c-erbB-2 expression with various pathological features, such as high cytological grade, large cell features, comedotype morphology and confluent neoplastic ducts, confirms the noticeable role of c-erbB-2 in the biological characterisation of breast DCIS.

About 61% of our cases showed positive staining for p53

Table 2. Spearman correlation coefficients between all considered 'markers'

	c-erbB-2	p53	ER	PR	CD44	cath-D	pS2	MLuC5
c-erbB-2	—	0.366*	−0.355†	−0.492*	−0.036	0.150	−0.369*	−0.242
p53	—	—	−0.065	−0.165	−0.222	0.161	0.056	−0.231
ER	—	—	—	0.627*	−0.193	0.146	0.468*	0.244
PR	—	—	—	—	0.027	0.145	0.504*	0.163
CD44	—	—	—	—	—	−0.067	−0.015	−0.077
cath-D	—	—	—	—	—	—	0.188	−0.068
pS2	—	—	—	—	—	—	—	0.289†
MLuC5	—	—	—	—	—	—	—	—

*$P < 0.01$; †$P < 0.05$.

ER, oestrogen receptor; PR, progesterone receptor; cath-D, cathepsin-D; MLuC5, 67-kDa laminin receptor.

Figure 6. Membrane immunostaining for MLuC5 in most neoplastic cells. Inner positive controls are provided by intervening stromal vasculature (magnification ×400; ABC method; haematoxylin counterstain).

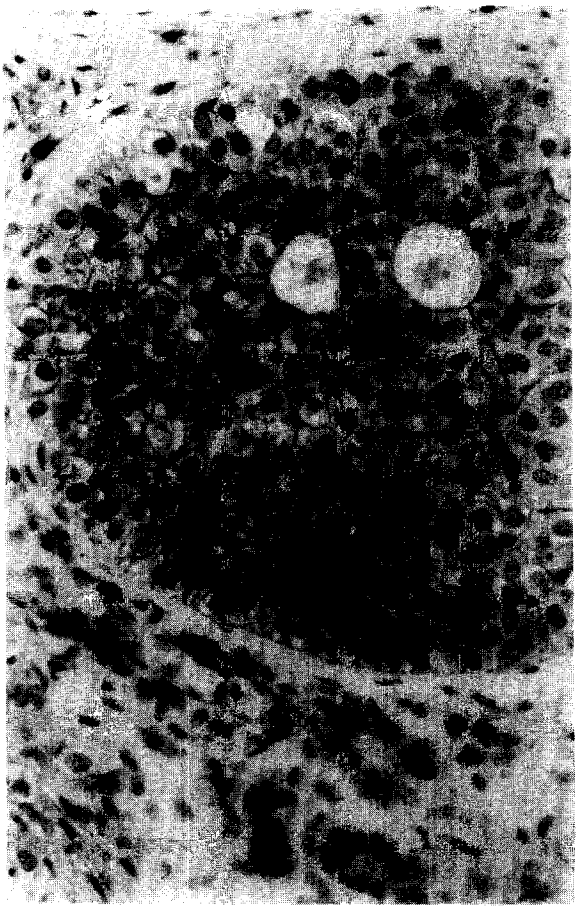

Figure 7. A neoplastic duct shows CD44-positive membrane cells. Inner positive controls are provided by scattered surrounding lymphocytes and plasma cells (magnification ×320; ABC method; haematoxylin counterstain).

protein, which is higher than 25% found by Poller and 40% found by Zafrani. In our study, immunopositivity of p53 correlated with high cytological grading, but the same could not be demonstrated with comedotype carcinoma and distribution of disease. In contrast with Poller and associates [25], we found a significant positive correlation between p53 positivity and c-erbB-2 overexpression. However, at variance with other authors [16], we were unable to show any significant association between p53 expression and negative ER and PR tumour status. The evidence of p53–c-erbB-2 correlation is consistent with the association of both markers with prognostically unfavourable histological features. Thus, as already suggested [16], the synchronous evaluation of p53 and c-

Table 3. Distribution of 'markers' according to grading and Spearman correlation coefficients

	Grading						
	G1		G2		G3		Spearman correlation coefficient and *P*-value
Markers*	%	*n/N*	%	*n/N*	%	*n/N*	
c-erbB-2 ⊕	27	3/11	60	15/25	69	9/13	0.316†
p53 ⊕	46	5/11	60	15/25	77	10/13	0.291†
ER ⊕	64	7/11	58	14/24	39	5/13	−0.249
PR ⊕	73	8/11	67	16/24	15	2/13	−0.396‡
CD44 ⊕	91	10/11	83	19/23	77	10/13	−0.131
cath-D ⊕	91	10/11	100	25/25	100	13/13	0.211
pS2 ⊕	64	7/11	71	17/24	46	6/13	−0.140
MLuC5 ⊕	82	9/11	92	23/25	62	8/13	−0.204

*Variations in the total number of cases is caused by unsatisfactory technique in some cases. †$P < 0.05$; ‡$P < 0.01$.
%, percentage of positive cases. *n/N*, number of positive cases/number of cases evaluated immunohistochemically. Abbreviations as in Table 2.

Figure 8. Focal immunostaining for cath-D is evident in some neoplastic cells, and in stromal hystiocytes (inner positive controls) (magnification ×400; ABC method; haematoxylin counterstain).

erbB-2 in DCIS may provide a useful insight into characterising the potential aggressiveness of the disease.

Determination of ERs and PRs in our series gave results similar to those reported in the literature, even though a frequency of positivity ranging from 32% to almost 80% has been assessed by some authors [15, 26, 27]. In the present study, hormone receptor positivity, namely PR, was significantly associated with low cytological grade. Previous studies have shown that expression of ERs and PRs is usually down-regulated in comedotype carcinoma as well as in extensive necrotic tumours [13–15], both displaying high cytological grade as well. Conversely, micropapillary and cribriform carcinomas, mostly characterised by low or intermediate cytological grade, are commonly ER and PR positive [13]. However, it has been shown that DCIS without invasive carcinoma was most frequently ER weak or negative, both in comedo and non-comedotype carcinomas [26], when compared to DCIS associated with invasive carcinoma. The reason for this finding is not clear, although it is possible that ER levels in DCIS may increase only when invasion develops in the surrounding tissue. As already seen in infiltrating breast carcinomas [28], in our DCIS cases, pS2 positivity was significantly correlated with ER and PR status. Accordingly, its detection may, therefore, be helpful in identifying patients eligible for hormonal therapy.

In the present study, cath-D positivity was not significantly associated either with other markers or with pathological features. Although this protein has been considered as a reliable prognostic marker of tumour progression in breast cancer patients [28, 29], its significance has to be further evaluated in non-infiltrating tumours. CD44, as marker of aggressiveness, has previously been evaluated in invasive breast cancer [30], but not in DCIS. Its inclusion in the present study was aimed at evaluating its expression on the latter, unexplored setting. To our knowledge, no conclusive studies are available on MLuC5 (67-kDa laminin receptor) expression in breast cancer [31]. Based on the interactions of this protein with basal membrane constituents (laminin), its assessment on DCIS would be valuable. Our results did not give significant correlative information on these markers. However, their overall highest frequency of expression in DCIS should be noted.

In conclusion, in this study positive correlations were found between c-erbB-2 and p53, and between PR and pS2. Positive correlations were also found between cytological grading and both c-erbB-2 and p53. An inverse relationship was seen between c-erbB-2 and a group of markers including ER, PR and pS2, as well as between cytological grading and PR. Finally, statistically significant association between c-erbB-2 and comedotype carcinoma and between c-erbB-2 and the distribution of disease in confluent neoplastic ducts was observed. Taken together, these findings further corroborate the relevance of c-erbB-2 and p53, on one side, and of ER, PR and pS2, on the other side, in biologically defining the morphological feature of DCIS.

Nevertheless, we have been unable to find any significant association between marker expression and recurrence rate. This may be due to the low number of cases in our study.

However, distinct trends between high recurrence rate and both p53 and c-erbB-2 overexpression were seen. From a clinical point of view, the figure of 22.2% of overall recurrence rate in our patients with DCIS treated with conservative surgery may indicate that a fraction of patients with DCIS of the breast should require more aggressive therapeutic approaches. In this regard, a biopathological characterisation of DCIS at the time of initial diagnosis might provide a valid aid in treatment decision making [32].

1. Swain SM. Ductal carcinoma in situ. *Cancer Invest* 1992, **10**, 443–454.
2. Peeters PHM, Verbtek ALM, Straatman H, *et al.* Evaluation of overdiagnosis of breast cancer in screening with mammography. *Int J Epidemiol* 1989, **18**, 295–299.
3. van Dongen JA, Holland R, Peterse JL, *et al.* Ductal carcinoma *in-situ* of the breast; second EORTC Consensus Meeting. *Eur J Cancer* 1992, **28**, 626–629.
4. Patchefsky AS, Schwartz GF, Finkelstein SD, *et al.* Heterogeneity of intraductal carcinoma of the breast. *Cancer* 1989, **63**, 731–741.
5. Lagios MD, Margolin FR, Westdahl PR, Rose MR. Mammographically detected duct carcinoma *in situ*. Frequency of local recurrence following tylectomy and prognostic effect of nuclear grade on local recurrence. *Cancer* 1989, **63**, 618–624.
6. Holland R, Peterse JL, Millis RR, *et al.* Ductal carcinoma in situ: a proposal for a new classification. *Semin Diagn Pathol* 1994, **11**, 167–180.
7. Bartkova J, Barnes DM, Millis RR, Gullick WJ. Immunohistochemical demonstration of c-erbB-2 protein in mammary ductal carcinoma in situ. *Hum Pathol* 1990, **21**, 1164–1167.
8. Ramachandra S, Machin L, Ashley S, Gusterson BA. Immunohistochemical distribution of c-erbB-2 in situ breast carcinoma—a detailed morphological analysis. *J Pathol* 1990, **161**, 7–14.

9. De Potter CR, Schelfhout AM. The neu-protein and breast cancer. *Virch Arch* 1995, **426**, 107–115.
10. Poller DN, Roberts EC, Bell JA, Elston CW, Blamey RW, Ellis IO. p53 protein expression in mammary ductal carcinoma *in situ*. Relationship to immunohistochemical expression of estrogen receptor and c-erbB-2 protein. *Hum Pathol* 1993, **24**, 463–468.
11. O'Malley FP, Vnencak-Jones CL, Dupont WD, Parl F, Manning S, Page DL. p53 Mutations are confined to the comedo type ductal carcinoma *in situ* of the breast. Immunohistochemical and sequencing data. *Lab Invest* 1994, **71**, 67–72.
12. van Agthoven T, Timmermans M, Foekens JA, Dorssers LCJ, Henzen-Logmans SC. Differential expression of estrogen, progesterone, and epidermal growth factor receptors in normal, benign, and malignant human breast tissues using dual staining immunohistochemistry. *Am J Pathol* 1994, **144**, 1238–1246.
13. Chaudhuri B, Crist KA, Mucci S, Malafa M, Chaudhuri PK. Distribution of estrogen receptor in ductal carcinoma *in situ* of the breast. *Surgery* 1993, **113**, 134–137.
14. Poller DN, Snead DRJ, Roberts EC, *et al.* Oestrogen receptor expression in ductal carcinoma *in situ* of the breast: relationship to flow cytometric analysis of DNA and expression of the c-erbB-2 oncoprotein. *Br J Cancer* 1993, **68**, 156–161.
15. Zafrani B, Leroyer A, Fourquet A, *et al.* Mammographically-detected ductal *in situ* carcinoma of the breast analyzed with a new classification. A study of 127 cases: correlation with estrogen and progesterone receptors, p53 and c-erbB-2 proteins, and proliferative activity. *Semin Diagn Pathol* 1994, **11**, 208–214.
16. Bobrow LG, Happerfield LC, Gregory WM, Springall RD, Millis RR. The classification of ductal carcinoma in situ and its association with biological markers. *Semin Diagn Pathol* 1994, **11**, 199–207.
17. Solin LJ, Yeh I-T, Kurtz J, *et al.* Ductal carcinoma in situ (intraductal carcinoma) of the breast treated with breast-conserving surgery and definitive irradiation. *Cancer* 1993, **71**, 2532–2542.
18. Hsu SM, Raine L, Fanger H. A comparative study of the peroxidase–antiperoxidase method and an avidin–biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. *Am J Clin Pathol* 1981, **75**, 734–738.
19. Craig Allred D, Clark GM, Molina R, *et al.* Overexpression of HER-2/neu and its relationship with other prognostic factors change during the progression of *in situ* to invasive breast cancer. *Hum Pathol* 1992, **23**, 974–979.
20. Armitage P, Berry G. *Statistical Methods in Medical Research*. Oxford, Blackwell Scientific, 1987.
21. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958, **53**, 457–481.
22. Mantel N. Evaluation of survival data and two new rank order statistic arising in its consideration. *Cancer Chemother Rep* 1966, **50**, 163–170.
23. Slamon DJ, Clark GM, Wong SG, *et al.* Human breast cancer: correlation of relapse and survival with amplification of HER-2/neu oncogene. *Science* 1987, **235**, 177–182.
24. Ottesen GL, Graversen HP, Blichert-Toft M, Zedeler K, Andersen JA. Ductal carcinoma *in situ* of the female breast. Short-term results of a prospective nationwide study. *Am J Surg Pathol* 1992, **16**, 1183–1196.
25. Poller DN, Hutchings CE, Galea M, *et al.* p53 protein expression in human breast carcinoma: relationship to expression of epidermal growth factor receptor, c-erbB-2 protein overexpression, and oestrogen receptor. *Br J Cancer* 1992, **66**, 583–588.
26. Bur ME, Zimarowski MJ, Schnitt SJ, Baker S, Lew R. Estrogen receptor immunohistochemistry in carcinoma *in situ* of the breast. *Cancer* 1992, **69**, 1174–1181.
27. Soomro S, Shousha S, Sinnet HD, *et al.* Oestrogen and progesterone receptors in screen-detected breast carcinoma: an immunohistological study using paraffin sections. *Histopathology* 1992, **21**, 543–547.
28. Hurlimann J, Gebhard S, Gomez F. Oestrogen receptor, progesterone receptor, pS2, ERD5, HSP27 and cathepsin D in invasive ductal breast carcinomas. *Histopathology* 1993, **23**, 239–248.
29. Gion M, Mione R, Dittadi R, *et al.* Relationship between cathepsin D and other pathological and biological parameters in 1752 patients with primary breast cancer. *Eur J Cancer* 1995, **31**, 671–677.
30. Joensuu H, Klemi PJ, Toikkanen S, Jalkanen S. Glycoprotein CD44 expression and its association with survival in breast cancer. *Am J Pathol* 1993, **143**, 867–874.
31. Gasparini G, Barbareschi M, Boracchi P, *et al.* 67-kDA Laminin-receptor expression adds prognostic information to intra-tumoral microvessel density in node negative breast cancer. *Int J Cancer* 1995, **60**, 604–610.
32. White J, Gustafson G, Levine A, *et al.* Outcome and prognostic factors for local recurrence in mammographically detected ductal carcinoma *in situ* of the breast treated with conservative surgery and radiation therapy (Abstract). *Int J Radiat Oncol Biol Phys* 1993, **27** (Suppl.), 31.