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Immunohistochemical study of *neu* protein overexpression in clinging in situ duct carcinoma of the breast

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Abstract. The expression of *neu* protein in 26 cases of clinging carcinoma (CC) of the breast was investigated. A distinction is made between two types of CC: one with pleomorphic nuclei (PN) and the other with monomorphic nuclei (MN). The PN type of CC overexpresses the *neu* protein in almost all cases (85.7%), its cells generally exhibit abundant cytoplasm and intraluminal necrosis is frequently observed. The MN type of CC does not overexpress the *neu* protein, exhibits bland cytological features and shows no necrosis. It is suggested that CC with PN is related to comedo-type carcinoma, while CC with MN is the forerunner of cribriform carcinoma in situ.

Key words: *Neu* protein – Breast cancer – Oncogene – In-situ duct carcinoma – Clinging carcinoma

Introduction

Clinging carcinoma (CC) was described by Azzopardi (1979) as a distinct type of in situ ductal carcinoma. It consists of a proliferation of a few layers of cells, which line the lumina of small ducts and acini. In a long term follow-up study it was shown that the relative risk of subsequent invasive carcinoma in CC is four times higher than with normal breast tissue (Eusebi et al. 1989). In the original description of Azzopardi (1979) two variants of CC were proposed. One was described as a subtype of comedocarcinoma in situ, and a second variant consisted of a proliferation of small atypical cells that appear to have originated in situ or have spread as a single well orientated layer of cells by a process of "creeping replacement" (Azzopardi 1979).

Recently the study of overexpression of the *neu* gene has provided interesting data on the biology of breast cancer. The overexpressed *neu* protein is a transmembrane protein, with a cell-external ligand-binding domain with tyrosine-kinase activity and is involved in signal transduction (Coussens et al. 1985). It has been shown that *neu* protein overexpression is a strong indicator of malignancy in the breast (De Potter et al. 1989a; Gusterson et al. 1988). In about 20% of primary invasive

Table 1. Clinging carcinomas: morphological and immunohistochemical results

Case N.	Nucleus	Nucleolus	N\C	Cytoplasm	Necrosis	*Neu* %
1)	MN	I	1	S	–	–
2)	MN	I	1	S	–	–
3)	MN	I	1	S	–	–
4)	MN	I	1	S	–	–
5)	MN	I	1	S	–	–
6)	MN	I	1	S	–	–
7)	MN	I	1	S	–	–
8)	MN	I	1	S	–	–
9)	MN	I	1	S	–	–
10)	MN	I	1	S	–	–
11)	MN	I	1	S	–	–
12)	MN	P	2	A	–	–
13)	PN	P	2	A	+	20
14)	PN	P	1;2	A	–	80
15)	PN	P	1;2	A	+	20
16)	PN	P	1;2	A	+	20
17)	PN	I	1;2	A	+	–
18)	PN	P	1;2	A	+	100
19)	PN	I	1	S	++	100
20)	PN	I	1	S	+	–
21)	PN	P	1;2	A	++	100
22)	PN	P	1;2	A	+	70
23)	PN	P	1;2	A	+	100
24)	PN	P	1;2	A	+	100
25)	PN	P	1;2	A	+	100
26)	PN	P	1;2	A	–	100

MN, Monomorphic; PN, pleomorphic; I, inconspicuous; P, Prominent; N\C, nuclear-cytoplasmic ratio; 1, N\C ratio = 1\1; 2, N\C ratio = 1\2; A, abundant cytoplasm; S, scanty cytoplasm; *Neu*% = percentage of *neu*-positive cells

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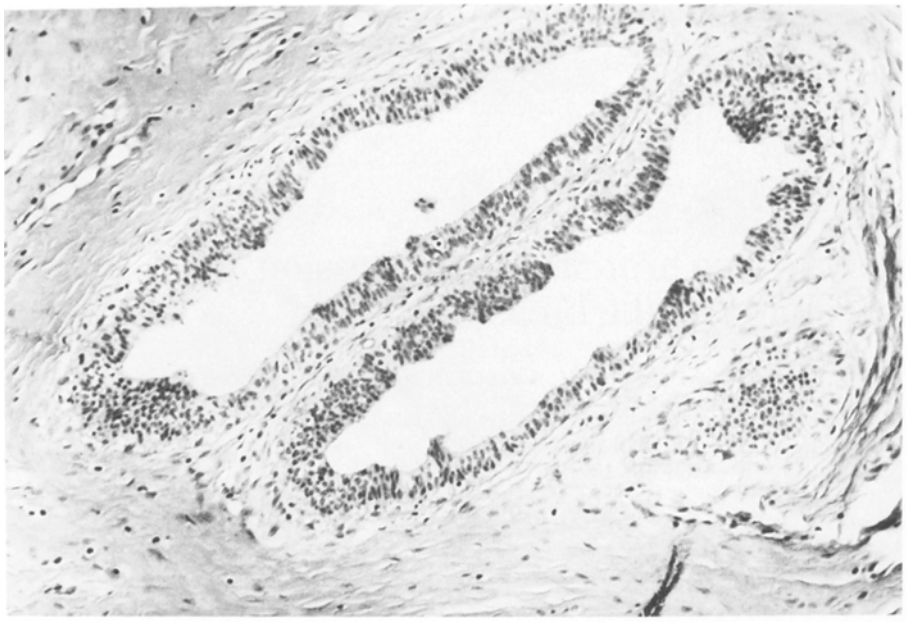

Fig. 1. Case 16, (PN case): two medium-sized ducts appear dilated and show irregular stratified epithelium which gives an irregular profile to the luminal border (H & E ×125)

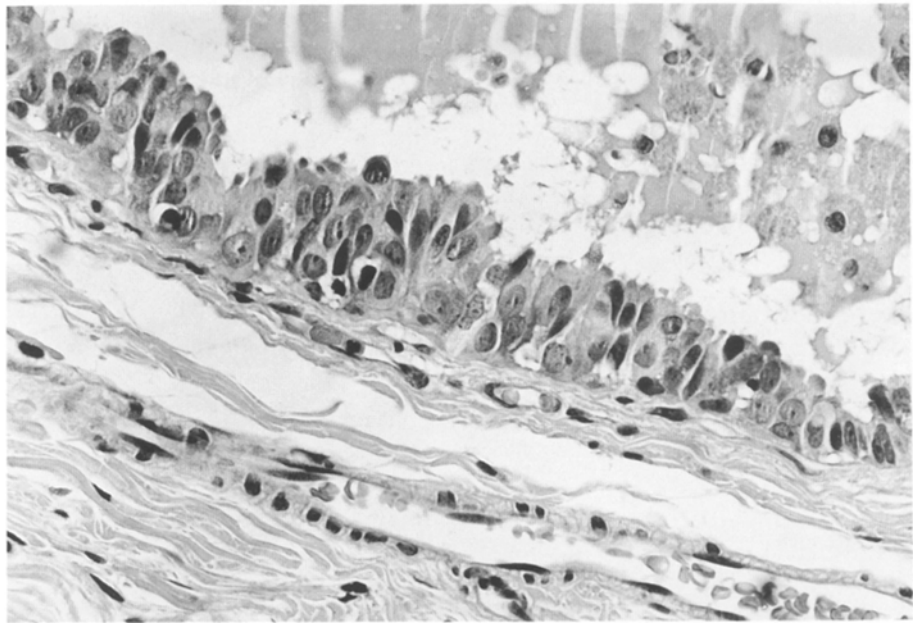

Fig. 2. Case 16, (PN case): cells are irregularly stratified. Nuclei show different shape and size (H & E ×250)

breast carcinomas amplification of the *neu* gene has been found (Slamon et al. 1987; Van de Vijver et al. 1988). Carcinomas showing *neu* protein overexpression are usually of ductal type and associated with an extensive intraductal component (Mooi and Peterse 1992). In purely intraduct carcinomas of large cell type, *neu* overexpression is found in about 70% of cases (Van de Vijver et al. 1988). In addition, most of the intraduct carcinomas which are positive for *neu* protein are of comedo-type (Bartkova et al. 1990). The purpose of this paper is to report on a study of the distribution of the *neu* protein in a series of 26 CC, using an anti-*neu* monoclonal antibody (mAb).

Materials and methods

Twenty-six cases of CC in situ were included in this study. Cases were retrieved from the routine material of the Department of Pathology, University of Bologna, at Bellaria Hospital and from the consultation files of one of us (VE). For the diagnosis of CC the criteria proposed by Azzopardi (1979) were used. Two cases were associated with an invasive duct carcinoma present in another breast quadrant, while the others were biopsies obtained after local excision without further treatment. The original sections were reviewed (average 3.4 blocks) and where changes of CC were present, the block was selected and new sections were cut.

In addition to the criteria proposed by Azzopardi (1979) several cytological criteria were taken into consideration. Nuclei were classified as pleomorphic (PN) when they had an irregular shape and

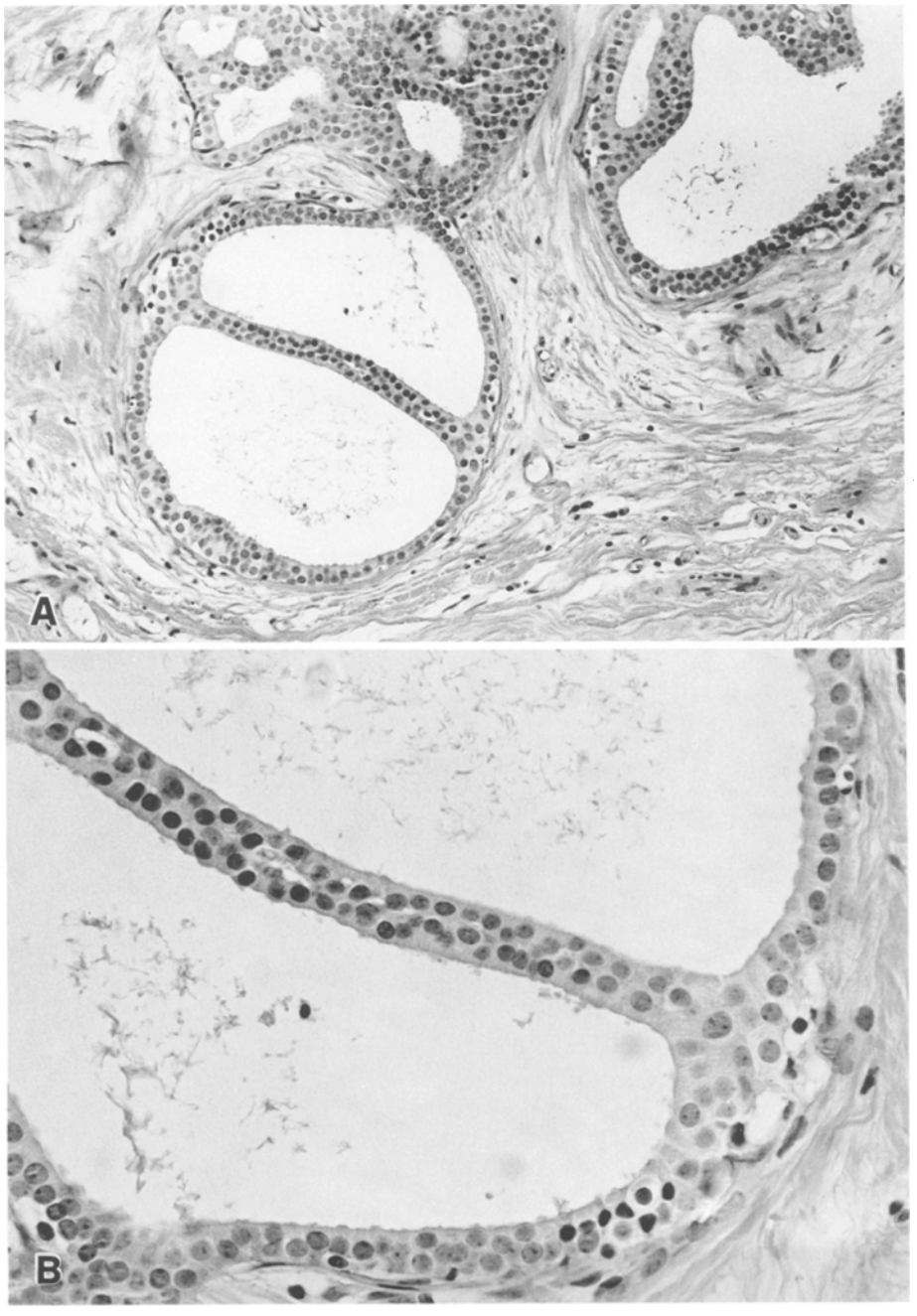

Fig. 3A. Case 9, (MN case): the cells lining the spaces are mono-bilayered. A trabecular bar divides the lumen in two portions (H & E ×125). **B** Cells with monotonous appearance, regular round nuclei with inconspicuous nucleoli (H & E ×250)

were of variable size, among the cells constituting a single lesion. Monomorphic nuclei (MN) were so classified when they had regular round to ovoid shapes and similar sizes in a given lesion.

Nucleoli were classified either as prominent when easily visible or as inconspicuous. The nuclear/cytoplasmic (N/C) ratio and the intraluminal presence of necrosis were recorded.

It is pertinent to note that cases were selected only when there was little or no intraluminal epithelial tufts/micropapillae. Types of CC showing numerous micropapillary fronds were not included into this study.

For the immunohistochemical investigations the avidin-biotin complex technique was applied as follows. Deparaffinized sections were immersed: in methanol containing 0.03% hydrogen peroxide for 20 min to block the endogenous peroxidase activities and incubated with 5% bovine serum albumin (BSA) for 30 min. A mouse mAb anti-*neu* antibody (50–100 µl per slide) (3B5, AB-3, Oncogene Science, Uniondale, USA) diluted 1:3000 in TRIS buffer saline (TBS) was then applied followed by 1% BSA for 30 min at room temperature, in a moist chamber. Biotinylated rabbit-anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) diluted at 1:400 in TBS were added and incubated for 30 min followed by the avidin-biotin complex (Dakopatts) according to the manufacturers instructions for 30 min. The chromogen used was 3-amino-9-ethyl-carbazole (Tissugnost Merk, Darmstadt, Germany) with 0.01% hydrogen peroxidase in acetate buffer pH 5.2, for 10 min. Between steps the slides were washed in buffer. All sections were dehydrated and mounted. Control sections were prepared by omission of the primary antibody. The cases were considered positive for *neu* overexpression only when membrane staining was observed, since cytoplasmic staining has been shown to be non-specific (De Potter et al. 1989b).

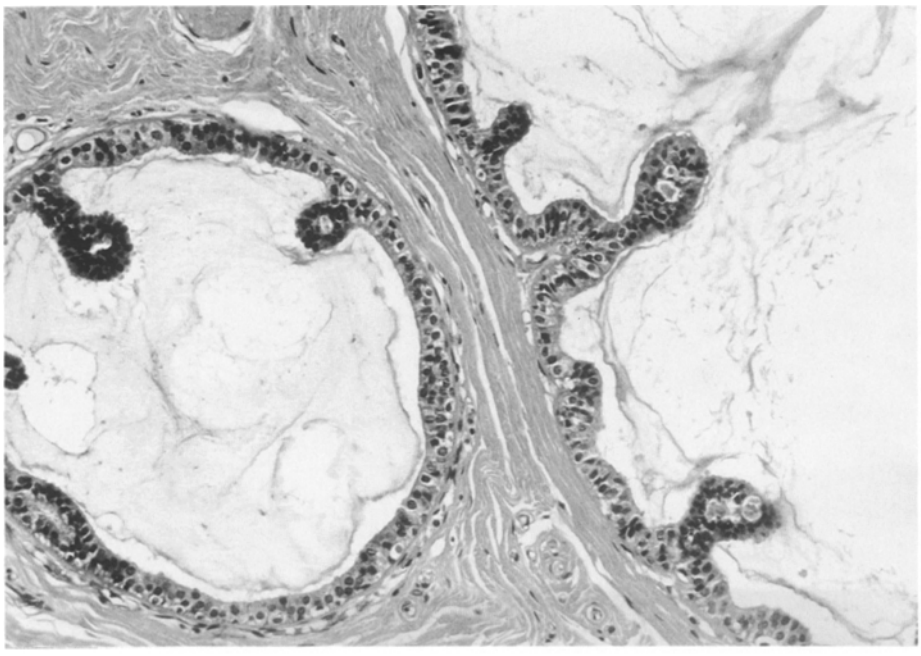

Fig. 4. Case 8, (MN case): Micropapillary clubs project into the lumina (H & E ×125)

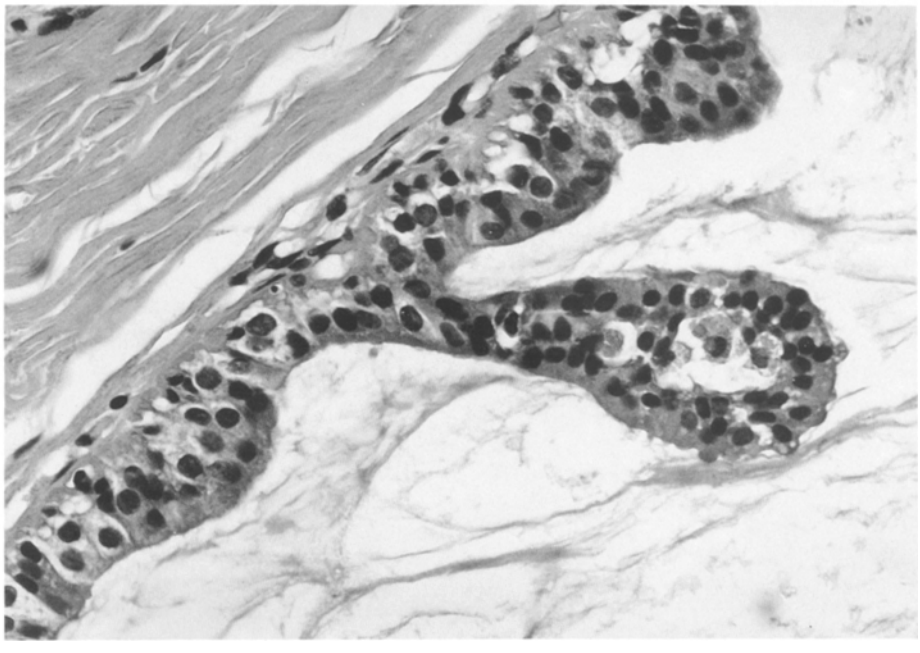

Fig. 5. Case 8, (MN case): the micropapilla is composed of a monotonous proliferation of cells similar to those lining the wall (H & E ×250)

Results

The age of the patients varied from 27 to 79 years (average 54.4). In 21 cases only a few small ducts and lobules were involved. In the remaining 5 cases numerous ducts and lobules displayed CC changes.

The results of the immunohistochemical investigation with 3B5 mAb and the main cytological findings are summarized in Table 1. PN were observed in no fewer than 14 cases (Figs. 1, 2). Cells with PN varied in number in different cases from 80 to 100%. In two of such cases nuclei were also grooved and several intranuclear cytoplasmic projections, similar to those seen in papillary carcinoma of thyroid, were seen. In 11 cases prominent nucleoli were present while in 3 cases nucleoli were small. The cytoplasm was abundant in most of neoplastic cells. Nevertheless some cells had scanty cytoplasm, consequently the nuclear/cytoplasmic ratio was very variable. Luminal necrosis was observed in 12 of these 14 PN cases. The epithelium was generally devoid of epithelial tufts\micropapillae (flat type of CC), as this was a criterion of selection. Nevertheless, occasional epithelial tufts\micropapillae were present in 8 of the 14 cases. Twelve cases belonged to the MN type and revealed nearly 100% of cells with a round to ovoid nucleus. The chromatin appeared finely dispersed and nucleoli were very small (Fig. 3). There was no luminal necrosis in these cases. Moreover, the cells exhibited scanty cyto-

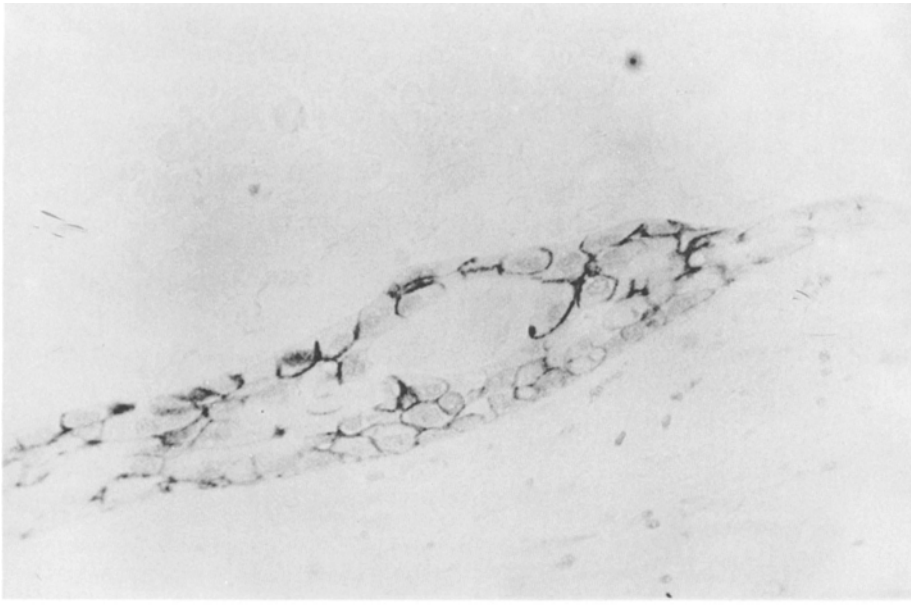

Fig. 6. Case 23, (PN case): the *neu* protein antiserum decorates the cell membranes of the neoplastic elements when the immunoperoxidase method is employed (ABC ×250)

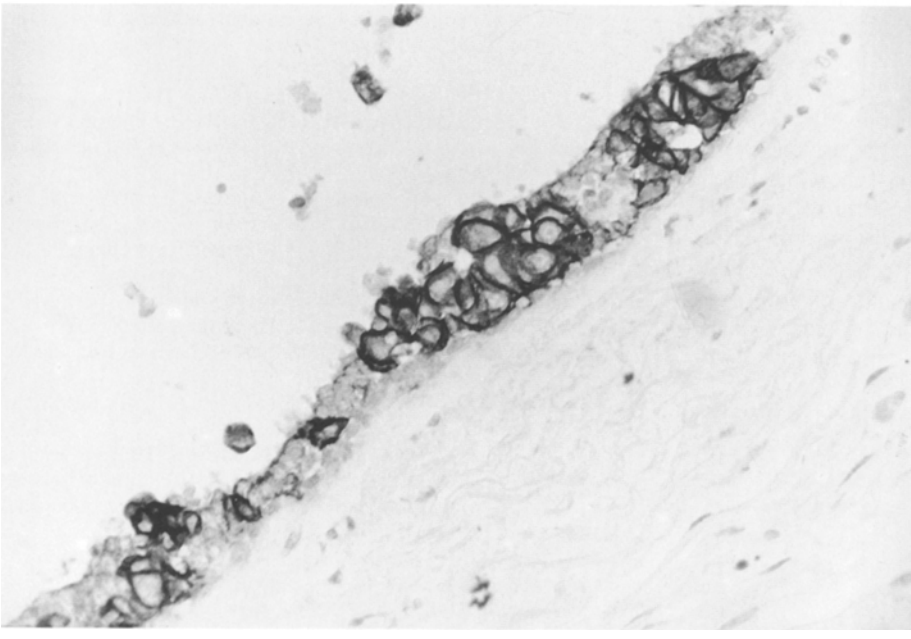

Fig. 7. Case 14: in some part of the ducts a mixed cell population is present. One is constituted by cells with PN, the other by elements with MN. Only the cells with PN overexpress the *neu* antigen, while the negative elements display MN (ABC ×250)

plasm and had a uniform nuclear\cytoplasmic ratio. Occasional epithelial tufts\micropapillae were present in no fewer than six of these patients (Figs. 4, 5). Only case 12 had prominent nucleoli and abundant eosinophilic and granular cytoplasm.

No age difference was found between the two groups (the mean age of patients with PN type of CC was 49.8, that of patients with MN type of CC was 54.3).

Of the 14 cases of CC of PN type, 12 showed cytoplasmic membrane staining for *neu* protein (Fig. 6). Positivity varied between staining of nearly the entire lesion (7 cases) to staining of about 20% of the total neoplastic proliferation. Usually only cells with PN nuclei, prominent nucleoli and abundant cytoplasm were stained. This was clearly shown in case 14, in which positive cells were intermingled with cells showing bland nuclei, small nucleoli and scanty cytoplasm (Fig. 7). One case deviated from this general rule. Case 19 showed numerous positive elements, despite the tumour cells having an inconspicuous nucleolus and scanty cytoplasm. Only 2 cases of a total of 14 CC of PN type failed to react with the *neu* antiserum, and, interestingly, these differed from the others morphologically as case 17 displayed small nucleoli and the tumour cells in case 20 had scanty cytoplasm as well as small nucleoli.

One of the invasive carcinomas, associated with an in situ component elsewhere, was immunohistochemically strongly positive for *neu* protein.

All 12 MN cases of CC were negative for overexpression of the *neu* protein.

Discussion

The CC type of breast lesion is not fully recognized as a variety of duct carcinoma in situ (DCIS) and is not included in the WHO classification of carcinoma in situ (WHO 1981). Here two types of CC have been delineated. One type consists of cells "clinging" to the walls of the small glandular structures with pleomorphic nuclei, prominent nucleoli, a variable N/C ratio, generally abundant cytoplasm, frequent luminal necrosis and exhibiting positivity with anti-*neu* protein antiserum. The second type has cells with round to ovoid nuclei of the same size and shape, a constant N/C ratio among neighbouring cells, generally scanty cytoplasm, no necrosis and no positivity for *neu* protein. This general statement applies to nearly all cases studied, the partial exceptions being cases 17, 19, 20 which differed by very small changes.

Van de Vijver et al. (1988) studied a small series of DCIS using an anti-*neu* antiserum. In this study *neu* overexpression was found mainly in comedocarcinomas. Bartkova et al. (1990) found strong correlation between *neu* overexpression and cases of DCIS which were characterized by large pleomorphic nuclei. Most of the Bartkova et al. cases (1990), like those of van de Vijver et al. (1988) were of comedo type, with the exception of one case which pertinently was described specifically as clinging in-situ duct carcinoma with large pleomorphic cells. By contrast cases which did not express the *neu* antigen were composed of cells with small nuclei and little variation in shape and size. The 12 cases of the present series which displayed membrane *neu* overexpression had pleomorphic nuclei. Conversely, with the exception of 2 cases, the immunologically negative cases showed cells having round to ovoid nuclei, without nucleoli and with little variation in shape and size.

It is concluded that CC can be separated cytologically in two subgroups which are in turn related to membrane *neu* overexpression or the lack of it, respectively. It appears that CC are analogous to the better known forms of DCIS among which the category of comedo carcinoma can be separated from the non-comedo types of DCIS on the basis of *neu* protein overexpression. Azzopardi (1979) had already separated CC into two categories on structural and cytological grounds, with one category representing a subtype of the comedo carcinoma.

On combined structural and immunological grounds, it seems, therefore probable that CC with PN are related to the full-blown classical comedocarcinoma, while CC with MN are the forerunners of other types of DCIS, including the cribriform variant.

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