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PIP/GCDFP-15 gene expression and apocrine differentiation in carcinomas of the breast

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Abstract The frequency and the significance of apocrine differentiation in carcinomas of the breast are uncertain, because of the lack of reliable and reproducible criteria for morphological diagnosis. The 15 kDa glycoprotein of cystic breast disease (GCDFP-15) is regarded as a specific functional marker of apocrine cells. Expression of the prolactin-inducible protein (PIP)/GCDFP-15 gene was investigated by Northern blot analysis and in situ hybridization in breast cancer cell lines and in an unselected series (33 cases) of primary carcinomas of the breast. On the same cases, histological assessment of apocrine differentiation and immunocytochemical detection of GCDFP-15 were also performed and correlated with follow-up data. The presence of PIP/GCDFP-15 mRNA was a feature of a relatively high number of cases, but was incompletely correlated with histological and immunocytochemical evidences of apocrine differentiation. Expression of the PIP/GCDFP-15 gene was significantly associated with relapse-free survival, and may represent a novel variable of functional and prognostic relevance.

Key words Breast neoplasms · Apocrine glands
Immunohistochemistry · Hybridization

Introduction

Carcinomas entirely composed of histologically recognizable apocrine cells form a minority of mammary carcinomas ranging between 1 and 4% (3, 8, 29). However, scattered areas of apocrine cells were found in about 10% of invasive cancers (8).

The histological definition of apocrine cells varies with different authors and this has led to great discrepancies in the literature on the incidence of this type of differentiation in carcinomas of the breast (1, 3, 10, 11, 14, 21). These discrepancies have prevented understanding of biological behaviour or the development of a selective therapeutic approach for these tumours.

The glycoprotein of 15 kDa, the major component of cyst fluid (GCDFP-15), represents an immunocytochemical marker of apocrine differentiation which is more objective and reproducible than morphology alone; several studies have delineated its presence and significance in breast cancer (12, 13, 18, 19, 20, 29, 34).

The gene coding for GCDFP-15 is localized in the long arm of chromosome 7 (24) and has recently been cloned (25). It is identical to the gene of the prolactin-inducible protein (PIP), independently described by Shiu and co-workers (23, 32) in the T47D cell line. Murphy et al. (22) reported on the expression of this gene in primary human breast carcinomas.

We recently devised a non-radioactive in situ hybridization (ISH) procedure to detect at cell level the expression of PIP/GCDFP-15 gene (27). In cases of cystic disease, ISH positivity correlated to a great extent with immunocytochemical (ICC) reactivity, but, in focal areas, discrepancies were observed since "pre-apocrine" cells (cells expressing the synthetic machinery but lacking both storage of the apocrine marker and related morphological features) were observed. Such findings in non-neoplastic lesions prompted an investigation in a series of breast carcinomas to establish presence, extent and cellular localization of PIP/GCDFP-15 gene expression, its relationship to morphology and to storage of the marker protein, as well as its relation to clinical data and evolution of the disease.

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Materials and methods

Thirty-three cases of primary human breast carcinomas were collected between 1987–90. Part of the tumour specimen was routinely processed by fixation in 10% formalin or Bouin's fluid and paraffin embedding while an adjacent tissue block was kept frozen at −80°C.

Serial sections were cut from the paraffin block of each case and stained in parallel with haematoxylin-eosin (H & E), immunocytochemistry and in situ hybridization.

For Northern blot analysis (NB), RNA was extracted from the frozen blocks. These were recruited from the bank of frozen tissues of the Department of Biomedical Science and Oncology of Torino.

Human breast cancer cell lines obtained from ATCC (Rockville, Md., USA), (cell lines BT20, MCF7, T47D, HS578T, MDA-MB231, SKBR3, BT549, MDA-MB361, BT474, ZR75.1) and cell line MCF10A from Michigan Cancer Foundation (Detroit, Mich., USA) were cultured under standard conditions. Stabilized cell lines obtained from R3230AC rat mammary carcinoma (30) and from SY2 mouse mammary cell carcinoma (16) were also tested. Total RNA was extracted from approximately $80–100\times10^6$ cells.

Histological examination, investigated in a double-blind procedure, took in consideration common morphological variables related to classification (3) and tumour grading (4), as well as the presence and extent of apocrine cell features, following the morphological criteria outlined by Eusebi and co-workers (8). Briefly these are: cells showing granular eosinophilic abundant cytoplasm intermingled with cells having clear foamy cytoplasm. Nuclei vary from hyperchromic to round to ovoid with prominent nucleolus.

Clinical data and follow up information were obtained in all cases. Chemotherapy was performed in patients with positive lymph-nodes.

The avidin biotin peroxidase method (ABC) was used for ICC (15). The primary antiserum was the polyclonal anti-GCDFP-15 diluted 1:5000 (kindly supplied by Dr. D. E. Haagensen, Sacramento, Calif., USA). Negative and positive controls (cases of cystic disease of the breast with apocrine cysts) were included with each batch of slides tested.

A 600 base pair cDNA encoding human PIP cloned into the pV21 bluescript plasmid vector (pPIP-8–3 cDNA clone, kindly supplied by Dr. R. Shiu and Y. Myal, Winnipeg, Manitoba, Canada) was used for ISH. The riboprobe was cleaved with the restriction enzyme XbaI and antisense RNA molecules were obtained using a T7 DNA polymerase and digoxigenin labelled nucleotide mixture (DIG RNA labeling kit, Boehringer, Mannheim, Germany).

The hybridization staining procedure has been described in detail elsewhere (27). Briefly, after proteinase K digestion, the slides were treated with the hybridization mixture containing the antisense digoxigenin labelled RNA probe. Following incubation, the non-specifically bound single strand RNA probe was removed by a RNAse digestion. The specifically bound hybridized probe was revealed using anti-digoxigenin antibodies conjugated with alkaline phosphatase. Colour was developed using nitro-blue-tetrazolium salt as a substrate. Slides were then dehydrated and mounted.

Appropriate positive and negative controls (colonic mucosa) were performed. Hybridization with non-specific riboprobes, such as that for somatostatin, produced negative results under identical hybridization conditions. RNAse digestion of tissue sections before the hybridization step caused the disappearance of the specific staining.

Only 26 of the 33 primary breast cancers were investigated for the PIP expression by ISH as 7 cases had been fixed in Bouin's solution which, in our experience, hampers results of ISH procedure.

For Northern blot analysis (NB) total RNA was extracted according to the guanidine thiocyanate-caesium chloride method (6) from the frozen tissue blocks and from breast cancer cell lines; the concentration of RNA was estimated by spectrophotometry and RNA degradation was monitored by agarose gel electrophoresis. The exact quantification of each line was monitored by laser densitometry of a photograph of the ethidium bromide-stained membranes with ultraviolet light (17). This approach was taken since variations in the expression of "house keeping" genes (e.g. actin) cannot be excluded in different tumours (33). NB, using P^{32}- labelled PIP riboprobe (see above), was performed on 10 μg of total mRNA of the 33 primary tumours and of the 13 breast cancer cell lines grown in vitro. As a positive control, 10 μg of total mRNA extracted from the T47D human breast cancer cell line, known to express the PIP gene (32), were run on each membrane. A detailed protocol for NB has been previously described (26). Briefly, the pre-hybridization, the hybridization and the washing temperature was 65°C. Blots were exposed to X-ray films with intensifying screens at −70°C for 18 h. Signal intensities of the specific bands were quantitatively assessed by densitometry (LKB 2202 ultroscan laser densitometer). Absolute values were expressed as arbitrary units of absorbency.

Statistical analyses were carried out with the SAS package (31). Survival function estimates were performed comparing the different levels of PIP mRNA expression (video-densitometric values) and considering both recurrences and deaths as relevant events. The statistical significance of the different parameters was evaluated by the log rank test, univariate and multivariate applications and by the χ^2 test (2).

Results

The main clinical-pathological data, the PIP/GCDFP-15 expression and the follow up of the 33 primary breast carcinomas investigated are reported in Table 1. The patients age ranged between 37 and 87-years-old (mean age: 62).

Histologically, 28 cases were infiltrating ductal carcinomas (IDC) with focal areas of in situ lesions which were prominent in 7 cases. Two cases were infiltrating lobular carcinomas (ILC) of the classical variety (28) and one was of the pleomorphic type (9). One featured mixed ductal and lobular patterns and one was a small cell neuroendocrine carcinoma (SCC).

One case (*n* 31) was classified as a classical apocrine carcinoma, while focal evidence of apocrine differentiation was detected in 12 other cases (see Table 1). Only one of these cases (*n* 27) was negative both by immunocytochemistry and by hybridization techniques for the production of GCDFP-15/PIP. By ICC, 21 (63%) of all cases were positive for the detection of the GCDFP-15 antigen: in 11 cases, the reaction was strong and diffusely positive while in 10 cases scattered nests of neoplastic cells were stained. In 3 cases (2 IDC and 1 ILC), only the in situ component appeared to store the GCDFP-15 antigen.

A good correlation was found between the GCDFP-15/PIP gene expression at protein and at mRNA level, investigated respectively by ICC and by hybridization procedures. Twelve (44%) out of the 26 cases tested by ISH were positive with a diffuse cytoplasmic staining in the same areas as seen by ICC (Fig. 1a and 1b). However, in occasional areas, the two staining methods were not totally superimposible (Fig. 2a–e). Five cases were focally positive only in ICC, while in one case scattered cells were positive by ISH alone.

By NB 17 cases out of the 33 investigated (50%) were characterized by the synthesis of variable amounts of the

Table 1 Clinical pathological data and PIP/GCDFP-15 expression in 33 cases of primary breast carcinoma. (*HIST/AP*, histological types and morphological evidences of apocrine differentiation; *IDC*, invasive ductal carcinoma; *ILC*, invasive lobular carcinoma; *SCC*, small cell carcinoma; *CLIS*, in situ lobular carcinoma; *CDIS*, in situ ductal carcinoma; *C*, classical type; *P*, pleomorphic type; *ER/PGR*, oestrogen and progesterone levels (RIA values); *ICC*, immunocytochemistry; *ISH*, in situ hybridization; +, weak positivity; ++, moderate positivity; +++, high positivity; °, ICC or ISH positivity limited to the in situ component; *NB*, Northern blot (densitometric values); *FU/MO*, follow up/months; *NED*, no evidence of disease; *DOD*, dead of disease; *AWD*, alive with disease.)

Number	HIST/AP	Grade	Stage	ER/PGR	PIP/GCDFP-15			FU/MO
					ICC	ISH	NB	
1	IDC/+	2	T2N1biii	39/60	+	ND	360	NED/57
2	IDC/+	2	T2N1bii	0/0	+	–	0	DOD/10
3	IDC/-	3	T2N0	17/160	+	ND	63	NED/43
4	ILC-CLIS/-	C	T3N1b	36/3	–	–	0	DOD/42
5	IDC-CDIS/+	2	T1N1	11/3	+	+	0	NED/41
6	IDC/-	2	T2N0	70/126	–	ND	197	NED/42
7	ILC-CLIS/-	C	T2aN0	20/90	°	–	0	DOD/2
8	IDC/-	2	T2N0	36/319	+++	++	812	NED/40
9	IDC/-	3	T2N1biii	21/0	–	–	0	DOD/27
10	IDC/-	2	T1cN1biv	31/35	+	–	0	DOD/19
11	IDC/-	3	T2N1bi	5/14	+++	++	403	NED/54
12	IDC-CDIS/+	2	T2N1bi	6/19	++	+	112	DOD/49
13	IDC-CDIS/+	3	T2N1	21/12	°	+	0	DOD/11
14	IDC-CDIS/-	2	T2Nx	ND/ND	°	°	0	DOD/22
15	IDC/-	2	T1aN0	86/26	+++	++	141	NED/53
16	IDC/-	2	T2N1biii	35/62	+	+	0	NED/54
17	IDC/+	3	T2N0	243/340	+++	+++	223	NED/63
18	IDC-CDIS/-	2	T2N0	0/0	++	+++	187	NED/70
19	IDC/-	3	T2N1	0/0	–	–	0	DOD/13
20	IDC/+	3	T2N0	392/519	+++	++	390	NED/72
21	SCC/-		T2N1	0/0	–	–	0	DOD/15
22	IDC/-	2	T1N1biv	ND/ND	–	–	0	NED/51
23	IDC/-	2	T1cN0	ND/ND	++	–	16	NED/53
24	IDC-CDIS/+	3	T1N1	139/11	++	ND	23	NED/56
25	IDC/+	2	T3N1M1	ND/ND	+	ND	12	AWD/50
26	IDC-ILC/-	2	T2N1	ND/ND	++	ND	71	NED/50
27	ILC/+	P	T3N1	20/32	–	–	0	NED/54
28	IDC/+	3	T2N1bi	14/10	+++	+	59	DOD/40
29	IDC/-	3	T2N0	83/2	–	–	0	NED/71
30	IDC-CDIS/-	2	T2N1biv	21/28	+	–	0	NED/50
31	IDC-CDIS/+	3	T2N0	26/28	+	+	381	AWD/62
32	IDC/-	3	T1N1b	27/7	–	ND	183	NED/45
33	IDC/+	2	T4bN1	19/18	+	–	0	DOD/55

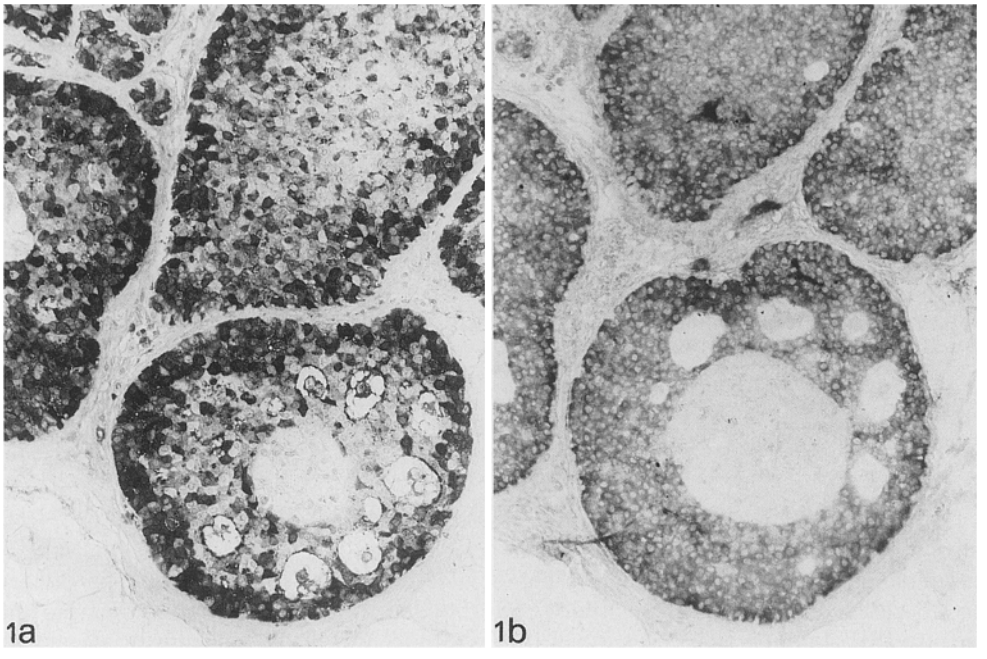

Fig. 1a, b Immunocytochemistry (**a**) and in situ hybridization (**b**) for PIP/GCDFP-15 performed on serial sections of a breast carcinoma (case 8). The reactions appear diffusely positive in an intraductal component of the tumour both at protein and at mRNA level (nuclei counterstained with methyl green; 200X)

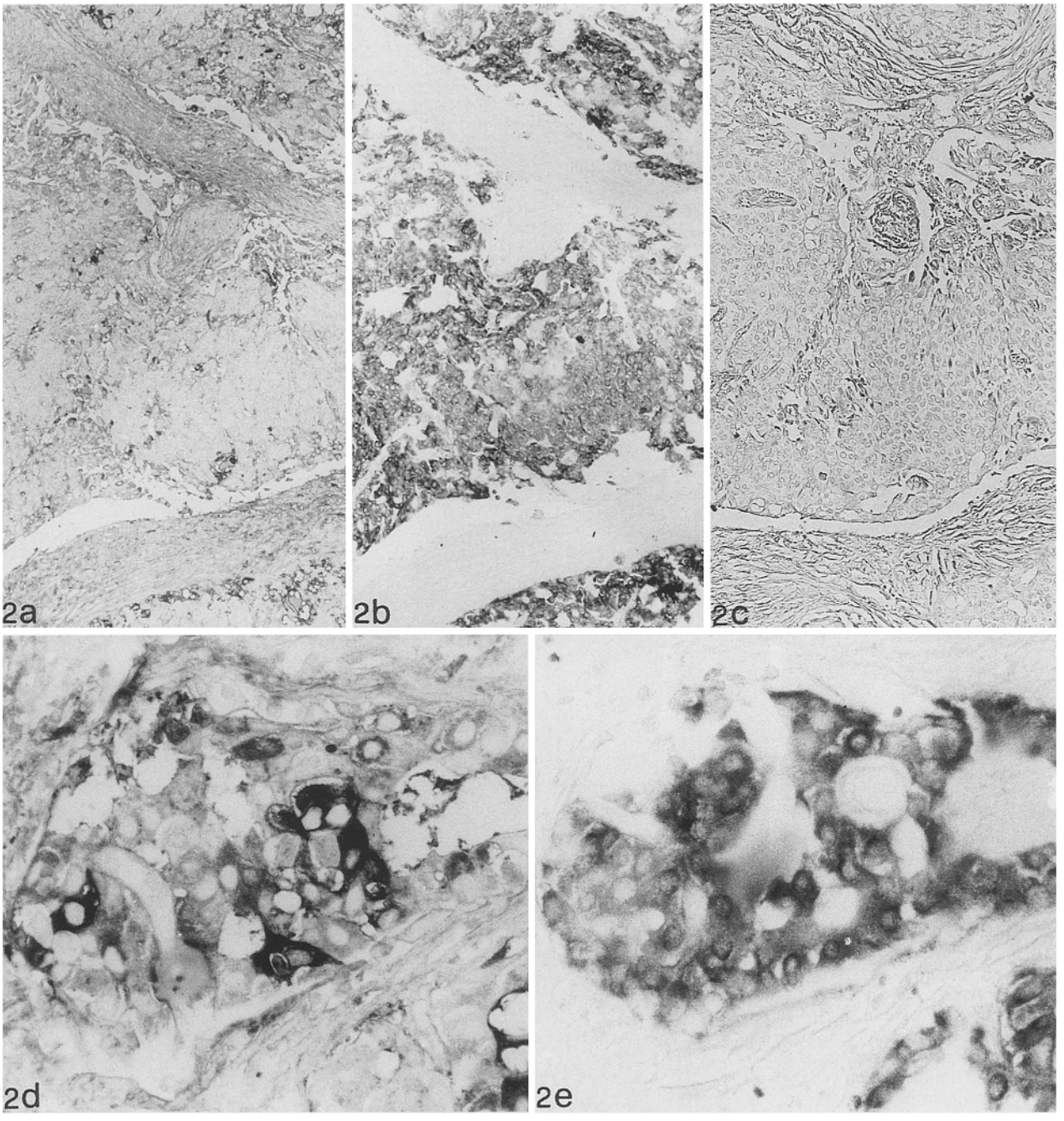

Fig. 2a–e The PIP/GCDFP-15 expression investigated on consecutive sections of a breast carcinoma (case 18) appears mostly negative at protein level by immunocytochemistry (**a**) while extensively positive at the mRNA level by in situ hybridization (**b**). (**c**) Lack of the specific staining due to RNAse digestion of the section before hybridization. At higher magnification, the focal distribution of the protein (**d**) contrasts with the higher number of ISH-positive cells (**e**) (nuclei counterstained with methyl green; **a**, **b** and **c**: 125X; **d** and **e**: 500X)

PIP mRNA molecules (Fig. 3). Six cases showed a strong PIP expression, others being characterized by a moderate (5 cases) or by a weak but appreciable positivity (6 cases). A strong NB positivity corresponded to a diffuse ISH staining. In 4 cases, focal ISH positivity was observed despite NB negativity; in one case (case 23), the opposite was found.

ICC and NB results were superimposible with some exception: in 6 cases where GCDFP-15 positivity was focal, NB was negative. In 2 cases (cases 6 and 32) moderate NB positivity was evident despite a completely negative ICC.

Three breast cancer cell lines (T47D, ZR75.1, BT474) out of the 13 investigated by NB were positive for the detection of the PIP mRNA.

The prognostic value of the PIP/GCDFP-15 expression, the presence of steroid receptors and histological evidences of apocrine differentiation were assessed by log rank univariate analyses (2) (see Table 2). The PIP

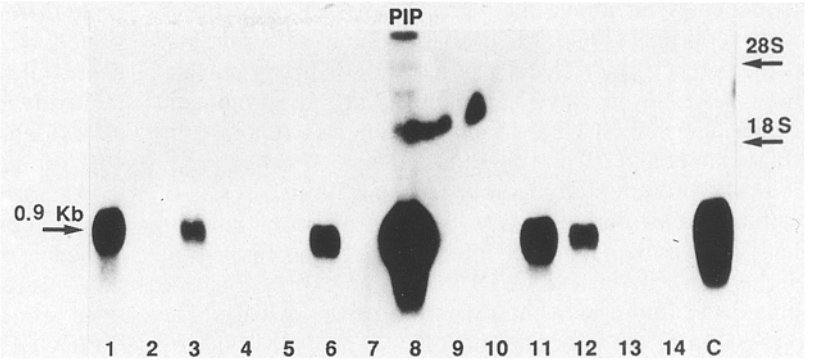

Fig. 3 Demonstration by Northern blot analysis of PIP mRNA in primary breast carcinomas. Lines number 1–14 (10 µg of total mRNA each one) correspond to case numbers of Table 1. Line labelled C represents the positive control (10 µg of total mRNA extracted by T47D breast cancer cell line). PIP mRNA migrates with 0.6 kb mRNA classes. The position of the 28S and 18S ribosomal RNAs are indicated on the right side of the figure

Table 2 Log-rank test (univariate analysis): the relapse-free interval is compared, for selected variables, below and above the median value (NB, Northern blot; *ICC*, immunocytochemistry; *ER*, oestrogen receptors; *PGR*, progesterone receptors; *AP*, morphological evidence of apocrine differentiation). All variables considered were associated in a statistically significant way with the clinical outcome, except the histological presence of apocrine differentiation. The most predictive factor, in the sense of a favourable neoplastic biological behaviour, was the synthesis of PIP mRNA as evaluated by Northern blot analysis ($P = 0.0075$)

Variable	p-value
NB/PIP	0.0075
ICC/GCDFP-15	0.0220
ER	0.0391
PGR	0.0415
AP	0.7117

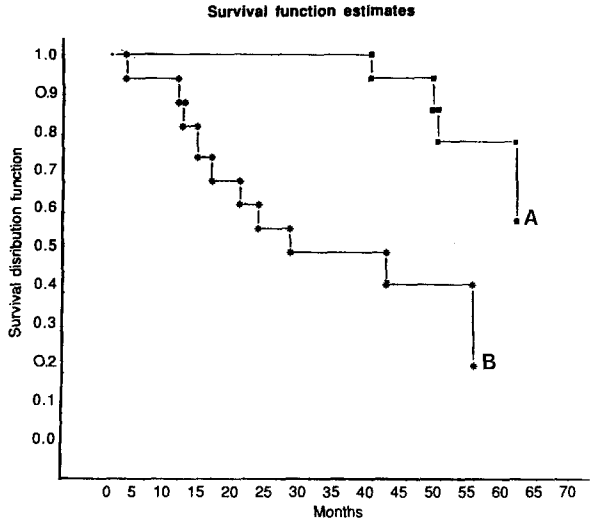

Fig. 4 Survival function estimates: relapse-free interval among PIP mRNA positive (**A**) or negative (**B**) breast carcinomas evaluated by Northern blot; cases alive with disease (*AWD*) were enclosed in the deceased group (*DOD*); a favourable clinical outcome of the PIP positive cases in comparison with the negative is appreciable ($P = 0.0075$)

mRNA expression, evaluated by NB, was highly predictive of the relapse-free interval ($P = 0.0075$) (see Fig. 4). The statistical significance was lower when examining the relapse-free interval related to ICC values and to levels of oestrogen and progesterone receptors (RIA values) (respectively $P = 0.0170$; $P = 0.0391$; $P = 0.0415$).

The presence of histological features of apocrine differentiation was not predictive of disease-free interval. By log rank multivariate analyses, only NB and ISH data were found to be associated with the clinical outcome in a statistically significant way (respectively $P = 0.0093$ and 0.0066). By the χ^2 test, a statistically significant association ($P = 0.04$) was found between absence of lymph-node metastases and PIP mRNA expression and also between the levels of progesterone receptors (RIA values) and the levels of PIP mRNA (video-densitometric values) ($P = 0.002$). Statistical significance was not reached comparing tumour size, levels of oestrogen receptors and PIP mRNA synthesis.

Discussion

Investigations on the incidence and significance of apocrine differentiation in breast carcinomas are of two types: those based on a purely histological definition of apocrine features (3, 29) and those supported by complementary techniques (9, 27). The observation by Haagensen and co-workers (13) that the glycoprotein of 15 kDa present in the breast cyst fluid was selectively produced in the apocrine epithelium provided a sensitive and specific functional marker for monitoring and defining apocrine differentiation in breast carcinomas especially in neoplastic conditions. Numerous authors have established the incidence and significance of apocrine differentiation in breast carcinomas, employing immunocytochemical procedures for localizing the GCDFP-15 marker (8, 12, 13, 18, 19, 20, 34). It was soon realized that this type of differentiation is detectable both in in situ and in invasive carcinomas (7). The apocrine differentiation is not restricted to invasive ductal carcinomas only, but it is shared by the pleomorphic variant of invasive lobular carcinomas (9).

The percentage of the GCDFP-15 positive cases varies in the literature, depending on the histological type and differentiation of the tumour series, on the antibody source and title used, and on the cut off of the percentage of positive cells considered to define the tumours as "positive". For all these reasons, the incidence of reported immunocytochemically positive tumours varies between 12 and over 50% (8, 18, 19, 20). Sixty-three per cent of the present cases were GCDFP-15 positive. This

incidence is far above the 12% of positive cases previously reported (8) and it is probably due to selection bias as this latter study reflects a series of 100 strictly consecutive cases of invasive carcinoma. Lack of prognostic significance of GCDFP-15 production was reported by Mazoujan et al. (20) and by Bundred et al. (5).

In the present study, conducted on a limited series of primary breast carcinomas with a follow up of not less than 40 months, we took into consideration both morphological criteria and GCDFP-15 gene expression, and related the findings to the evolution of the disease. The expression of the apocrine marker was analysed both by immunocytochemical detection of the stored protein, and by using Northern blot analysis and a novel in situ hybridization procedure devised by our group (27) to detect presence of the specific mRNA.

Morphology alone, independently investigated in a double-blind evaluation, recognized extensive (one case) or focal evidence of apocrine differentiation in 13 out of the 33 cases. This is a much higher figure than that observed previously in a consecutive series of 100 cases of breast carcinomas (8). This is again to be referred to a selection bias in the present series. However the main purpose of the present study was to compare the different criteria used to recognize apocrine differentiation. Expression of apocrine markers was found by immunocytochemical and/or hybridization analysis in 23 cases, while morphological criteria recognized apocrine features in 12 of those cases. This confirms our previous conclusions (8) on the poor sensitivity and relatively high specificity of purely histological criteria in defining apocrine differentiation in carcinomas.

Expression of the PIP/GCDFP-15 gene in breast carcinomas had already been investigated by NB by Murphy et al. (22). These authors detected presence of the specific mRNA in 61% of a series of 51 carcinomas and found positive correlation with oestrogen receptors, a known indicator of favorable prognosis. Using the same PIP riboprobe, half of our cases were positive, to a high (11 cases) or low degree (6 cases) of expression. Results of ISH procedure were comparable to those obtained by NB but, even in cases negative by the latter procedure, we observed occasional ISH positivity in minute foci of in situ ductal carcinomas.

The lack of a total correlation between the immunocytochemical and the hybridization procedures seems to be a recapitulation of what was observed in cases of cystic disease of the breast (27). In one case (n 13) neoplastic cells endowed with the mRNA PIP synthetic machinery appeared to be unable to produce and/or store the specific protein. In the opposite case (cases 7, 10, 23, 30, 33), immunocytochemically positive but ISH negative cells might either have lost the synthetic ability or, as an alternative, have passively absorbed the protein.

We observed a highly significant association between presence of PIP/GCDFP-15 mRNA as revealed by both NB and ISH analysis and relapse-free interval. The finding might indicate a more favourable evolution of apocrine-differentiated breast cancers, but caution is suggested by the relatively low number of cases, by the short follow up and by some selection bias, including the relatively high number of carcinoma showing histological evidence of apocrine differentiation and the fact that 33% of our patients were node negative.

At variance with Murphy et al. (22) we have not found a correlation between apocrine gene activation and presence of oestrogen receptors, but a positive correlation was found with progesterone receptors.

In conclusion, it seems that the synthesis of the PIP/GCDFP-15 apocrine marker may be indicative of a functional differentiation which is not constantly related to structural patterns. The purpose of this paper was to present a further morphological definition of apocrine differentiation of breast carcinoma, using the new genetic approaches. The clinical impact of this novel prognostic variable and its apparent independence from other known predictors of evolution need to be further investigated.

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