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Bilateral apocrine carcinoma of the breast

Molecular and immunocytochemical evidence for two independent primary tumours

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Abstract Apocrine carcinoma is an uncommon variant of breast cancer. The frequency of bilaterality in patients who have apocrine carcinoma in one breast is not significantly different from that for bilateral mammary carcinomas in general, but bilateral apocrine carcinomas are very uncommon. We report on a bilateral apocrine carcinoma of the breast in a 74-year-old woman. The apocrine differentiation in both tumours was confirmed by the positivity of the cytoplasmic granules for PAS after diastase digestion and immunoreactivity for GCDFP-15 and sialyl-Tn. The tumour in the right breast showed immunohistochemical expression of p53, and a mutation was demonstrated by PCR-SSCP; the tumour in the left breast was negative for p53 on immunohistochemistry, and no mutation was found at the molecular level. c-erbB2 expression was not detected in the right tumour but there was overexpression (at the cell membrane) in the left tumour. Both tumours were aneuploid: the right tumour displayed multiple stemlines, whereas the left tumour had a triploid profile. Using the fluorescence in situ hybridization technique we demonstrated that both tumours displayed chromosome 17 polysomy and numerical abnormalities of chromosome 1, polysomy in the right and monosomy in the left tumour. We conclude that the two tumours are probably independent, as are most bilateral carcinomas of the breast.

Key words Breast apocrine carcinoma · Bilateral breast cancer · p53 · c-erbB2 · Chromosomal abnormalities

Introduction

Bilateral breast cancer is a well-known and relatively common clinicopathological finding. The reported frequency of bilaterality in breast carcinomas ranges from 3% to 12%, varying with differences in study design, patient selection and sampling techniques [8]. Bilateral tumours may be synchronous or metachronous; women with breast carcinoma have an increased risk of developing carcinoma in the contralateral breast. The risk of developing a carcinoma in the contralateral breast in the first decade after the primary treatment has been estimated at 5–10% [4, 11]. There is some evidence that the frequency of bilaterality is higher in some specific histological types of breast cancer, e.g. lobular carcinoma [4].

Apocrine carcinoma is an uncommon variant of breast cancer, being composed entirely or almost entirely of histologically recognizable apocrine cells: cells showing granular, eosinophilic and abundant cytoplasm and enlarged and pleomorphic nuclei with prominent nucleoli [1, 6, 10]. The apocrine differentiation can be corroborated by additional studies: the presence of PAS-positive cytoplasmic granules, immunoexpression of the 15-kDa glycoprotein of cystic breast disease (GCDFP) [1, 8, 10] and sialyl-Tn [13], and the demonstration of empty vesicles and osmiophilic granules at the ultrastructural level [8]. Apocrine differentiation may also be defined by genotypic features, such as the presence of mRNA for PIP/GCDFP-15 [10]. According to Eusebi et al.[6], the detection of PIP/GCDFP-15 mRNA by in situ hybridization is more specific than immunohistochemistry in establishing the apocrine nature of a given tumour, owing to the possibility of false positivity caused by passive diffusion of the protein GCDFP-15.

The frequency of bilaterality in patients who have apocrine carcinoma in one breast is not significantly different from that of bilateral breast carcinomas in general: an occasional patient has nonapocrine carcinoma in her contralateral breast [7], but the presence of apocrine carcinomas in both breasts is extremely rare [12]. The occurrence of a bilateral apocrine breast carcinoma raises a particularly interesting biological and clinical question: are we dealing with two separate primary tumours or a metastasis from one breast to the other?

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We report the morphological, immunohistochemical and molecular findings of a bilateral apocrine carcinoma of the breast in a 74-year-old woman.

Case report

A 74-year-old woman was admitted because of bilateral breast tumours. Fine-needle aspirations were performed and malignancy was diagnosed in both breasts. Bilateral mastectomy with axillary dissection was undertaken. In the right breast, the tumour nodule measured 4 cm and there were 3 among 12 lymph nodes with metastases. In the left breast, the tumour had 3 cm and the 14 lymph nodes isolated from the axilla were free of metastases. The patient does not have family history of breast cancer.

Material and methods

Light microscopy

Tissue samples were fixed in buffered 10% formalin, dehydrated and embedded in paraffin; 5-µm sections were cut and stained with haematoxylin and eosin and periodic acid-Schiff.

For immunostaining the avidin-biotin-peroxidase complex (ABC) method was used. The primary antibodies used were: oestrogen receptor, clone ER1D5, diluted 1:100 (Dakopatts, Glostrup, Denmark); progesterone receptor, clone PR10A9, diluted 1:20 (Immunotech, Marseille, France), cytokeratin CAM 5.2, diluted 1:5 (Becton-Dickinson, Belgium), MIB-1, diluted 1:200 (Immunotech), c-erbB-2, diluted 1:200 (Dakopatts); p53 protein, clone DO-7, diluted 1:50 (Dakopatts), GCDFP-15 diluted 1:10,000 (Signet Labs., Mass.); and sialyl-Tn, diluted 1:8 (Dakopatts). For sialyl-Tn and GCDFP-15 the incubation was done at room temperature with no requirement for microwave processing. A negative control test for the immunostaining was carried out by substituting the primary antibody with a mouse myeloma protein of the same subclass and concentration of the primary antibody. As a positive control we used sections from a case of invasive breast carcinoma known to express ER, PR, CAM 5.2, MIB-1, c-erb-B2, p53, sialyl-Tn and GCDFP-15. Tumour cells showing distinct brown nuclear staining were interpreted as positive for ER, PR and p53. Cases with 5% or more tumour cell nuclei showing p53 immunostaining were scored as positive [16]. Tumour membrane immunoreactivity, either homogeneous or heterogeneous, was used as the sole criterion of c-erbB-2 overexpression. MIB-1 was scored by counting 1000 cells [13].

For DNA measurements paired sections were cut from each paraffin block selected, and were dewaxed and rehydrated. One slide from each block was stained by the Feulgen technique (acid hydrolysis 5 N hydrochloric acid at room temperature for 60 min). The other section was stained with haematoxylin-eosin. A pathologist (F.S.) identified and marked microscopic fields for image analysis on the HE-stained slides. The DNA measurements were performed using a TV-based image analysis system (Ahrens System, Bargteheide/Hamburg, Germany). It is based on a Nikon light microscope (plan objective ×40) equipped with a video-CCD camera (Panasonic 500) connected to a microcomputer.

The cytophotometric measurements of stained cell nuclei were performed at a wavelength of 546±10 nm. Areas corresponding to those outlined on the haematoxylin-eosin-stained sections were identified in the Feulgen-stained sections. The lymphocyte nuclei from each section served as the internal controls. From each case 200 nuclei from the tumour and 50 control nuclei were measured. DNA histograms were generated by plotting nuclear optical density of Feulgen-stained DNA against the number of nuclei. For each case the G0/G1 peak was identified visually, and the mean, standard deviation (SD) and coefficient of variation (CV) values were calculated. The control CV provided an indication of overall precision of the imaging technique. Aneuploid peaks were those exceeding the internal control lymphocyte (diploid) G0/G1 peak mean by 2SD. The percentage of nuclei exceeding 5n DNA content, as defined by the control cell 2n DNA content, was also calculated.

Using a modification of Auer's classification scheme, the histograms were classified into "euploid" (types I and II) or "aneuploid" (types III and IV) [16, 17]. A type I histogram is characterized by a single distinct peak in the diploid or near-diploid region of normal cells with the "tumour" G0/G1 DNA mean value within 2SD of the control cell G0/G1 DNA mean values. Type II populations show either a distinct modal value in the tetraploid or near-tetraploid region or have two well-defined peaks around the 2n and 4n regions, presumably representing overlapping nuclei or cells arrested in the G2 phase. Type III histograms have a tumour G0/G1 peak exceeding the control cell G0/G1 peak mean by 2SD of the control cell peak. Type IV histograms show very pronounced and irregular aneuploidy, with DNA amounts ranging from 2n up to values in excess of 6n or even 8n [3].

In the in situ hybridization study, a number of consecutive 4-µm sections were cut from the formalin-fixed, paraffin-embedded tissue and were mounted on silanized slides. The last section was stained with haematoxylin-eosin and was examined to confirm the presence of the lesion, which was then outlined. We used alpha-satellite probes, conjugated to biotin and specific for centromeric regions of chromosome 1 (D1Z5) and 17 (D17Z1), purchased from Oncor (Gaithersburg, Md.). Deparaffinized sections were treated with 0.8% pepsin (Sigma, St. Louis, Mo.) in 0.2 M HCl (Merck, Darmstadt, Germany) for 15 min at 37°C, in a pre-warmed humidified chamber. To improve protein digestion, the slides were previously soaked in 1 M sodium thiocyanate (Merck) for 10 min at 80°C. After protein digestion the slides were again dehydrated and allowed to air dry. A probe mixture was prepared by combining 3 ml of biotinylated probe with 60 ml of Hybrisol VI (Oncor), providing a hybridization medium with 65% formamide, 10% dextran sulphate, 2× SSC (pH=7.0). Then 15 ml of probe mixture was applied to each slide, covered with a 22×30 mm glass coverslip and sealed with rubber cement (Oncor). Probe and target DNA were simultaneously denatured at 80°C for 10 min in a wet chamber. Hybridization was performed for 16 h at 37°C. After hybridization rapid washing was performed by transferring slides to a glass coplin jar containing prewarmed 1× SSC, at 72°C, in a waterbath, and soaking for 5 min without agitation. The slides were then transferred to 1× PBS at room temperature for 2 min. Detection was performed with fluorescein-labelled Avidin (Oncor). Propidium iodide/antifade (Oncor) was used for counterstaining. In each hybridization session, a negative control was performed using a probe mixture made consisting of Hybrisol VI (Oncor) without any added probe.

Signal evaluation was performed by one pathologist in an epifluorescence Olympus BH-2 microscope, using the Olympus B-L0910 filter set. For all cases, at least 200 nuclei were evaluated and the number of signals per nucleus was recorded. Eligible nuclei were located in regions with good hybridization performance (signals present in more than 80% of cells) and presented well-defined nuclear borders, negligible auto-fluorescence and no overlapping.

DNA was extracted from microdissected normal and tumour cells obtained from formalin-fixed, paraffin-embedded tissue sections. Five slices of each paraffin block were cut. The first and last slice were HE-stained for histological confirmation, and the three intermediate slices were microdissected for further DNA extraction using a phenol-chloroform extraction method.

Mutations in the *p53* gene were analysed by single-stranded conformational polymorphism (SSCP) with primers covering exons 2-3 to 11. PCR conditions using each of these primers were 45 cycles of 94°C – 1 min; 58°C – 1.5 min; 72°C – 2 min, followed by an extension period of 10 min at 72°C. A volume of 3 µl of the PCR product was diluted 1:1 in a gel-loading buffer (20% formamide/ 0.25% xylene cyanol/ 0.25% bromophenol blue), denatured at 94°C for 5 min and run in a polyacrylamide gel. Electrophoresis was performed on a 6% neutral polyacrylamide gel with 10% glycerol at 60 W for 4 h at 20°C. The gel was dried and exposed to X-ray film for 12–24 h.

Results

The histological sections from the tumour of the right breast revealed an infiltrating carcinoma without an intraductal component, with a predominantly solid pattern composed of cells with abundant eosinophilic and granular cytoplasm. The nuclei were enlarged and pleomorphic and contained large, prominent and usually eosinophilic nucleoli. The mitotic count was 15 per 10 high-power fields at the periphery of the tumour (field diameter: 0.63 mm, Leitz Diaplan microscope ×40 magnification). In the tumour of the left breast we observed a predominantly intraductal carcinoma with some small foci of invasion, made up of cells with characteristics similar to those of the contralateral tumour: abundant and eosinophilic granular cytoplasm and enlarged nuclei with prominent nucleoli (Fig. 1). The intraductal architecture showed a solid, micropapillary and cribriform pattern. The invasive areas were similar to those observed in the infiltrating carcinoma of the right breast.

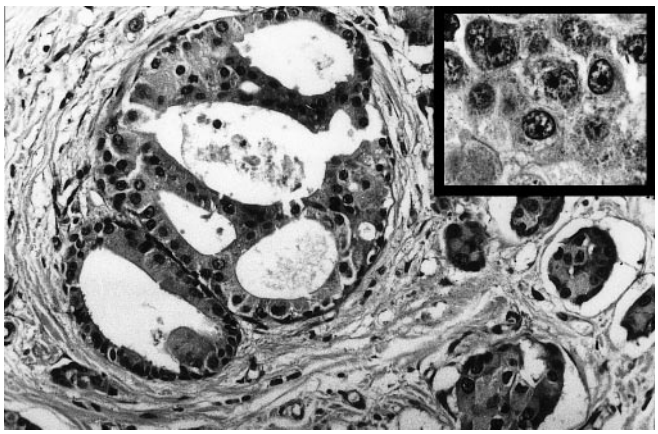

Fig. 1 Predominantly intraductal apocrine breast carcinoma of the left breast. Note the cells with abundant, eosinophilic and granular cytoplasm and the large nuclei with prominent nucleoli (*insert*), which are similar to those observed in the right tumour. HE, ×250

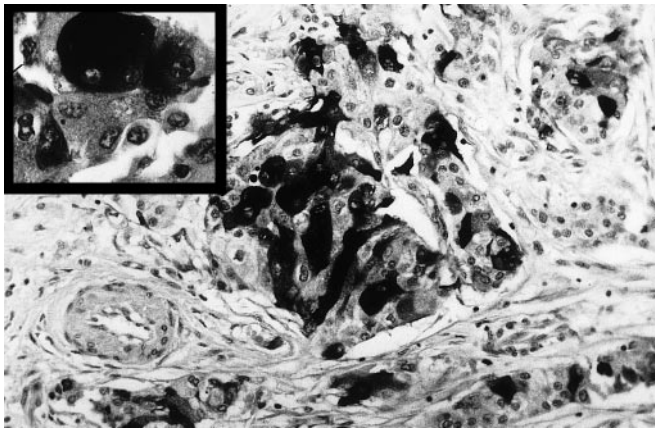

Fig. 2 Sialyl-Tn positivity in the cytoplasm of the apocrine neoplastic cells. Immunohistochemistry, counterstaining with haematoxylin, ×250

The cytoplasm of both tumours showed granules intensively stained by PAS after diastase digestion.

Immunohistochemical staining showed positivity in both tumours for cytokeratin (CAM 5.2), GCDFP-15 and sialyl-Tn (Fig. 2). The two latter were positive in more than 85% of the tumour cell population. Both tumours were negative for ER and PR. The right one was positive for p53 and negative for c-erbB2, and the left revealed the opposite pattern, being p53 negative and c-erbB2 positive. The proliferative index measured by MIB-1 staining was 25.4% on the right side and 20.3% on the left side.

Table 1 summarizes the findings recorded in both tumours in the cytometric and molecular studies. Both tumours were aneuploid on DNA cytometry but displayed different histogram patterns. The right tumour showed an Auer's type IV histogram with several stemlines, whereas the left tumour had an Auer's type III histogram. The results of FISH studies using the D17Z1 probe evaluated in 200 nuclei showed gains for chromosome 17 in both tumours. Nuclei with three or more distinct, bright hybridization signals made up the major part of the cell population. Using the D1Z5 probe for chromosome 1 in 200 nu-

Table 1 Comparative immunohistochemical, cytometric and molecular findings in the apocrine breast carcinomas of both breasts

Feature	Right tumour	Left tumour
Immunohistochemical		
CAM 5.2	Positive	Positive
GCDFP 15	Positive	Positive
Sialyl Tn	Positive	Positive
ER and PR	Negative	Negative
P53	Positive	Negative
c-erbB2	Negative	Positive
MIB-1 (Proliferation index)	25.4%	20.3%
DNA ploidy	Aneuploidy (Auer's type IV histogram)	Aneuploidy (Auer's type III histogram)
FISH		
Chromosome 1	Polysomy	Monosomy
Chromosome 17	Polysomy	Polysomy
p53 mutations (SSCP)	Positive(exon 7)	Negative

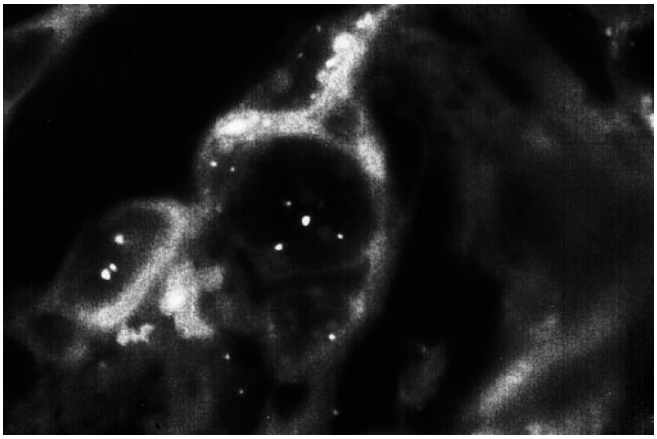

Fig. 3 FISH in interphase nuclei from the right tumour using D1Z5 probe showing gains in chromosome 1. Note the nuclei with three or more signals. Some of the signals are visible out of the plane of focus

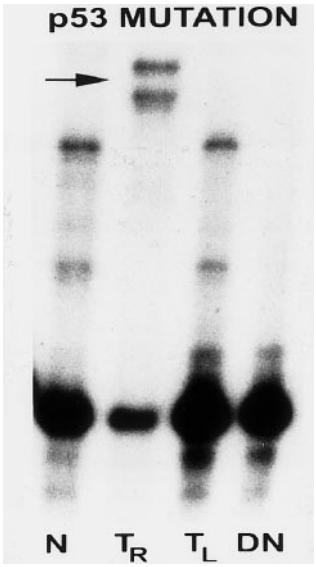

Fig. 4 SSCP-PCR analysis of exon 7 of *p53*, showing a mutation in the right tumour. Note the left tumour's normal pattern of migration on gel (similar to control)

clei, we observed multiple signals (polysomy) in the nuclei from the right tumour. The left tumour showed monosomy for chromosome 1, with more than 90% one-signal nuclei (Fig. 3).

Mutant *p53* was detected only in the right tumour. The mutation was found in exon 7 (Fig. 4).

Discussion

Both tumours fulfilled the cytomorphological criteria outlined by Eusebi et al. [6] as defining a breast carcinoma as apocrine. Briefly, these are: a tumour composed entirely of cells with abundant granular, eosinophilic cytoplasm and enlarged and pleomorphic nuclei with prominent nucleoli. Apocrine change may occur in about 10% of invasive breast cancers, but the diagnosis of apocrine carcinoma should be reserved for tumours composed entirely or almost entirely of recognizable apocrine cells [1, 6, 10]. The frequency of apocrine carcinoma in different series ranges between 1% and 4%, and this variability probably results from the inconsistency of the diagnostic criteria. Apocrine carcinomas can have the same architecture as other mammary carcinomas, differing only in the cytological appearance. In the present case, the tumour on the right is an infiltrating apocrine carcinoma (grade II), and the tumour on the left is an infiltrating apocrine carcinoma with a predominantly intraductal component exhibiting a solid, micropapillary and cribriform pattern. The apocrine differentiation in both tumours was confirmed by the positivity of the cytoplasmic granules for PAS after diastase digestion and for GCDFP-15 [5, 6, 10]. Recently, we demonstrated that the apocrine epithelium had a pattern of simple mucin-type glycosylation, which is specific and distinct from that of the normal breast epithelium. There is strong and very frequent expression of Tn and sialyl-Tn in apocrine metaplasia [14]. This finding probably reflects an abnormal differentiation rather than a premalignant event, and does not mean that an apocrine metaplasia is a precursor lesion of breast carcinoma. Our findings in this case show that apocrine carcinomas also stain very intensely for sialyl-Tn, thus reinforcing the notion that this antigen can be used as a marker of apocrine differentiation in benign and malignant breast lesions.

The incidence of bilaterality in invasive breast carcinoma is about five times that in the general population, and lobular histology, presence of in situ carcinoma and a family history of breast cancer are indicators of increased risk for bilaterality [8]. Bilateral apocrine carcinomas are very rare, and their occurrence raises the question of whether two separate primary tumours are concerned, or metastasis from one breast to the other.

Previous studies based on classic cytogenetic evaluation suggest that most bilateral breast carcinomas arise independently [11]. In our case, the morphological, immunohistochemical, cytometric and interphase cytogenetics findings also suggest the presence of two independent carcinomas. Although both carcinomas showed apocrine differentiation, the tumour on the right side was a predominantly invasive carcinoma with axillary metastases, whereas the other was a predominantly intraductal carcinoma, with few foci of invasion and without metastases. Although Pandis et al. [11] have suggested on the basis of cytogenetic studies that the in situ pattern of breast carcinomas may arise through a metastatic process, this is highly debatable and does not fit in with the data observed in our cases.

The expression of c-erbB2 and p53 was different in the two tumours: the right tumour had immunohistochemical expression of p53 and a mutation was demonstrated by PCR-SSCP, whereas the left tumour was negative for p53 on immunohistochemistry and no mutation was found at molecular level. The role of p53 in the pathogenesis of bilateral breast carcinomas out of a familial context is rather

controversial. The presence of *p53* mutations in both tumours is rare compared with series of unilateral breast carcinomas [2]. The present case reinforces these findings. It is interesting to point out that some authors have suggested that alterations to the *p53* gene are more frequent in specific types of breast carcinomas, including the apocrine type [9]. The discrepancy of c-erbB2 immunoreactivity in both tumours also suggests that they are independent of each other. The c-erbB2 expression observed in the present case fits in with our previous findings of the higher frequency of c-erbB2 positivity in invasive carcinomas with an extensive intraductal component than in purely invasive carcinomas [15]. These findings are thought to support the putative involvement of c-erbB2 activation in the intraductal breast cancer cell motility [15], but discussion of such a possibility is far beyond the scope of this paper.

The cytometric profile was also different in both tumours. Although both were aneuploid, the right tumour displayed a histogram with multiple stemlines, whereas the left tumour had a triploid profile. The frequent maintenance of the histogram profile in in situ and invasive components of breast cancer as well as in their metastases [15, 17] reinforces the assumption that one is dealing with two independent tumours in this case.

Finally, the differences in numerical alterations of chromosome 1 found by FISH analysis in the two tumours are in accordance with previous studies that demonstrated that most bilateral breast carcinomas display significant cytogenetic differences and do not originate from a metastatic process [11].

The present case is an example of a rare situation in breast pathology: an apocrine carcinoma presenting as a bilateral tumour. We think we have obtained enough immunohistochemical, cytometric and molecular evidence to claim that despite their histological and cytological similarities, the two tumours are probably independent.

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