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Chromogranin A and B Gene Expression in Carcinomas of the Breast

Correlation of Immunocytochemical, Immunoblot, and Hybridization Analyses

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Chromogranins (Cg) are regarded as specific neuroendocrine (NE) markers in cells and tumors. Expression of CgA and CgB genes has been demonstrated by correlative immunocytochemical, immunoblotting,* in situ *hybridization, and Northern blot procedures in seven argyrophilic breast carcinomas, while eight control cases of ductal carcinomas, not otherwise specified, were negative. A high degree of correlation was observed between the various techniques revealing CgA and/or CgB gene expression at different levels; minor discrepancies might be related to tumor heterogeneity or to technical factors. The present study, confirming previous investigations, establishes NE differentiation in a group of human breast cancers. The identification of this type of tumors, especially by testing chromogranin(s) production, appears to be of both biologic and clinical interest. (Am J Pathol 1990, 136:319—327)

Evidence for the existence of neuroendocrine (NE) differentiation in human breast carcinomas is presently based on microscopic data. Histologic similarity to carcinoid tumors described in other organs and the argyrophilia of some breast cancers were first noticed by Feyrter and Hartmann,[1] and by Cubilla and Woodruff.[2] Their observations were confirmed and extended by others,[3-11] while ultrastructural investigation of these tumors revealed the presence of membrane-bound, dense cytoplasmic bodies, interpreted as endocrine secretory granules[3,6,11,12] or as milk proteins.[13,14]

The nature of these tumors was not resolved by immunohistochemical analysis: panels of antibodies against known polypeptide hormones were tested, but positive cells could only be found sporadically.[12,15] In accordance with these results, no functional endocrine syndrome associated with this type of carcinoma has been clearly observed, although descriptions of single cases associated with Cushing's syndrome or with biogenic amine production are reported.[16-18]

More rewarding was the immunocytochemical detection of chromogranin A (CgA), a polypeptide with no known hormonal function, but regarded as a specific NE marker. A monoclonal against CgA,[19] which proved positive in a series of endocrine tumors and cells in different organs, revealed chromogranin-containing cells in a series of argyrophilic carcinomas of the breast, thus confirming their NE differentiation.[8,9] Recently, chromogranin was localized in dense cytoplasmic granules in these tumors by immunoelectron microscopy.[11] The present study is a further step forward in defining chromogranin production and establishing the existence of a NE type of breast cancer.

Materials and Methods

Fifteen cases of breast carcinoma were collected from the Department of Pathology, University of Turin, Italy. Six of these were selected from a larger series of breast tumors that were processed according to standardized criteria and with immunocytochemical stains: all six cases

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Table 1. *Antibodies Used for Immunohistochemistry (IHC) and/or Immunoblotting (IB)*

Chromogranin	Type of test	Type of reagent	Source	Dilution in IHC	Reference
A (Phe-5)	IHC	Monoclonal	Enzo Biochem, New York, NY	1:500	—
A (LK2H10)	IHC	Monoclonal	Dr. R. V. Lloyd, Ann Arbor, MI	1:100	11
A (human)	IHC, IB	Polyclonal	our lab	1:200	23
B (human)	IHC, IB	Polyclonal	our lab	1:100	23
B (DK 21) (synthetic)	IHC, IB	Polyclonal	Dr. Hogue-Angeletti, Philadelphia, PA	1:1000	29
secretogranin II	IB	Polyclonal	our lab	—	23

positively stained for Grimelius silver impregnation[20] and NE markers. The remaining nine control cases were part of a consecutive series of breast carcinomas sent for intraoperative diagnosis and were entered in this study with no previous knowledge of their histo- and immunochemical characteristics. Specimens from the 15 cases were divided in five parts within 20 minutes of surgical resection, as follows:

- one block was frozen in liquid nitrogen and serial cryostat sections were obtained for intraoperative diagnosis and receptor analysis;
- one adjacent block was fixed in Bouin's solution and processed for conventional histology, histochemistry (Grimelius method), and immunohistochemistry for chromogranins;
- two blocks (approximately 5 mm^3) were snap frozen in liquid nitrogen and then processed for either immunoblot analysis or Northern blotting;
- one block of three cases (numbers 3, 4, and 10) was fixed in freshly prepared precooled (4 C) 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 hours; the samples were then submerged at 4 C in 30% sucrose-0.02% DEPC(diethyl pyrocarbonate)-PBS for 5 hours and finally embedded and frozen in OCT medium at −75 C; cryostat sections were cut and used for *in situ* hybridization.

Immunocytochemistry

Serial sections from the Bouin-fixed specimen were tested with different antibodies to chromogranins. The type of reagent, source, and working dilution are listed in Table 1.

The reactions were detected by the avidin-biotin-peroxidase complex (ABC) procedure, according to Hsu et al.[21] Endogenous peroxidase activity was blocked according to Heyderman and Neville.[22]

Immunoblot Analysis

After freezing, tumor samples were lyophilized, cut into small pieces, homogenized with distilled water in a Potter-Elvejhem homogenizer, and boiled for 5 minutes according to the procedure outlined elsewhere;[23] insoluble material was sedimented by centrifugation at 120,000*g* for 45 minutes and the supernatant was lyophilized and resuspended in a small amount of distilled water. For immunoblotting, aliquots of the heat-stable proteins underwent one-dimensional sodium dodecylsulphate electrophoresis with an acrylamide gradient range of 10% to 17%, or two-dimensional electrophoresis.[24,25] Immunoblots were obtained using the antisera in Table 1 and following a modified Burnette protocol.[26,27] Proteins were measured according to Lowry et al[28] after protein precipitation with 2% perchloric acid. The specificities of chromogranin A and B and secretogranin II antibodies have been reported elsewhere.[23,29]

Probes

Antisense RNA probes for chromogranin A (CgA) were generated by transcription of a linearized RNA expression vector, p GEM 2, containing a 528 bpDNA sequence of the bovine chromogranin A gene,[30] using ^{32}P-labeled UTP (Amersham, Buckinghamshire, England); the probes (specific activity of 8×10^8 cpm/mg probe) were used within 2 days for *in situ* hybridization and Northern blot analysis. The probe was supplied by Dr. Eiden (NCI, Bethesda, MD).

A 39-residue oligonucleotide[31] was synthesized following assignments for the C-terminal region of human chromogranin B (CgB) gene sequence.[32] The probe was labeled using ^{32}P-labeled dATP (Amersham) by a 3′-tailing reaction (Amersham), and used within 2 days for *in situ* hybridization and Northern blot analysis (specific activity of 5×10^9 cpm/mg probe). Oligonucleotides, based on amino acid sequence of rat chromogranin A,[33] were labeled as described above (specific activity of 10^9 cpm/mg probe); the probes were used within 2 days for *in situ* hybridization. Both oligonucleotides were supplied by Dr. Fischer-Colbrie (NCI, Bethesda, MD). The specificity of the probes was controlled on different tissues. Positive hybridization signal was found in adrenal medulla, parathyroid gland, anterior pituitary gland, pheocromocytoma,

parathyroid adenoma, bronchial carcinoid, and Merkel cell tumor. No specific reaction was observed in adrenal cortex and striated muscle.

Northern Blot Analysis

Total RNA was extracted according to the guanidine thiocyanate-cesium chloride method.[34] The RNA extract was quantified by spectrophotometric analysis. RNA degradation was monitored by agarose gel electrophoresis; only those samples in which the ratio of 28S and 18S RNA exceeded 3:1 were processed further. Ten micrograms total RNA of each case were separated on denaturing gels containing formaldehyde[35] and transferred onto nylon membranes by vacuum blotting.

CgA ^{32}P-labeled c-RNA probes were used after prehybridization at 68 C overnight (prewarmed buffer: 50% deionized formamide, 5×SSC pH 7.0, 10× Denhardt's, 50mM NaH_2PO_4/Na_2HPO_4 pH 6.5, 1% SDS, denatured ssDNA 1 mg/ml buffer); hybridization was performed at 70 C overnight (prewarmed buffer: 50% deionized formamide, 5×SSC pH 7.0, 2× Denhardt's, 25mM NaH_2PO_4/Na_2HPO_4 pH 6.5, 1% SDS, denatured ssDNA 100 mg/ml buffer, 2×10^6 cpm probe/ml buffer); washing was done at 70 C in a prewarmed 2×SSC, 0.1% SDS solution for 30 minutes, followed by two 30 minutes washing steps in a prewarmed 0.2×SSC, 1% SDS solution. Blots were exposed to X-ray films with intensifying screens at −70 C for 17 hours.

CgB ^{32}P-labeled oligonucleotides were used after prehybridization at 42 C for 2 hours (prewarmed buffer: 5× SSC, 20mM NaH_2PO_4/Na_2HPO_4 pH 7.2, 7% SDS, 10× Denhardt's, 10% Dextran sulphate, denatured ssDNA 100 mg/ml buffer); hybridization was done at 42 C overnight in the same buffer containing 2×10^6 cpm probe/ml buffer; washing was performed at 42 C in a prewarmed solution of 3×SSC, 10× Denhardt's, 5% SDS, 25mM NaH_2PO_4/Na_2HPO_4 pH 7.2 for 1 hour, followed by a second washing in a prewarmed 1×SSC, 1% SDS solution for 30 minutes. Blots were exposed to X-ray films with intensifying screens at −70 C for 19 hours.

In Situ *Hybridization*

A detailed protocol for *in situ* hybridization using c-RNA probes has been published.[36] We used the same procedure with oligonucleotide probes except in some steps: immersion in 0.1M glycine and in 0.3% Triton X was omitted; the hybridization buffer was modified as follows: 4×SSC, 50% deionized formamide, 1× Denhardt's, 1mM EDTA pH 8, denatured ssDNA 100mg/μl buffer, t-RNA 100 mg/μl buffer, 10% Dextran sulphate, 10 mM DTT, to which probe (5×10^5 cpm/10 μl buffer) was added. The RNAse step was omitted and washing was performed at 42 C. Hybridization sites were detected by autoradiography, employing liquid emulsion (Kodak NTB-2; Rochester NY., USA); slides were exposed for 5 days, developed, counterstained (H&E), and viewed under light- and dark-field illumination.

Controls for Hybridization Analysis

Hybridization with nonspecific vector sequences and sense RNA probes, generated from the same vector construct, produced negative results, under identical hybridization conditions, both in Northern blot and *in situ* hybridization.

Results

The 15 cases were all infiltrating breast carcinomas. The histology of the six selected cases and of the single chromogranin-positive case (number 14, see below) of the consecutive series were characterized by solid (alveolar or trabecular) patterns and lack of glandular structures and of marked cytological atypias.

The remaining eight cases of the consecutive series were ductal carcinomas, not otherwise specified.

Immunohistochemistry

To test content of chromogranin A and B we used specific monoclonal antibodies and antisera: similar results were obtained, although the former showed lower sensitivity, as revealed by relatively lower number of positive cells in single cases. The six selected Grimelius-positive cases were strongly or moderately reactive for chromogranin A (CgA) in five cases, while case 3 gave negative reaction. Cases 5 and 6 were CgA positive when tested with the antiserum, while negative with the monoclonals. Four cases were positive for chromogranin B (CgB) (Figure 3a), while cases 1 and 2 were negative. Case 14 from the consecutive series was positive for CgA but not for CgB (see Table 2). The rest of the consecutive series were negative for all immunocytochemical tests.

Immunoblot Analysis

Tumor extracts were subjected to one dimensional electrophoresis and immunoblotting with antisera to chromogranins. Cases were considered positive only where an

Table 2. *Comparison of Immunohistochemistry (IHC), Immunoblot Analysis (IBA), and Northern Blot Analysis (NBA) for Chromogranins in 15 Breast Carcinomas*

Case no.	Criterion of case selection*	Chromogranin A			Chromogranin B		
		IHC	IBA	NBA	IHC	IBA	NBA
1	selected	+	nd	+	−	nd	−
2	selected	+	+	+	−	−	−
3	selected	−	−	−	+	−	+
4	selected	+	−	+	+	−	+
5	selected	±	+	−	+	+	+
6	selected	±	+	nd	+	−	nd
7	random	−	−	−	−	−	−
8	random	−	−	−	−	−	−
9	random	−	−	−	−	−	−
10	random	−	−	−	−	−	−
11	random	−	−	−	−	−	−
12	random	−	−	−	−	−	−
13	random	−	−	−	−	−	−
14	random	+	+	+	−	+	−
15	random	−	−	−	−	−	−

* Selected: chosen because argyrophilic and positive for NE markers; random: randomly drawn from a consecutive series of breast cancer, as "controls" (see text).
nd, not done (material not available).
±, cases weakly positive with polyclonal serum, while negative with both monoclonals.

immunoreactive CgA band, moving like adrenal CgA, was detected. An immunoreactive component, corresponding in electrophoretic behavior to adrenal CgA, was present in four cases. Figure 1 shows representative results in cases 2 and 6. Cases 5 and 14 (not shown) contained very small amounts of CgA and a faster-moving immunoreactive band. Two-dimensional immunoblotting of these latter cases could not identify such band as a breakdown product of CgA, but confirmed the presence of immunoreactive spots in a position corresponding to that obtained with adrenal CgA in all four cases. The other 10 cases were negative. One case (number 1) was not tested because material was not available.

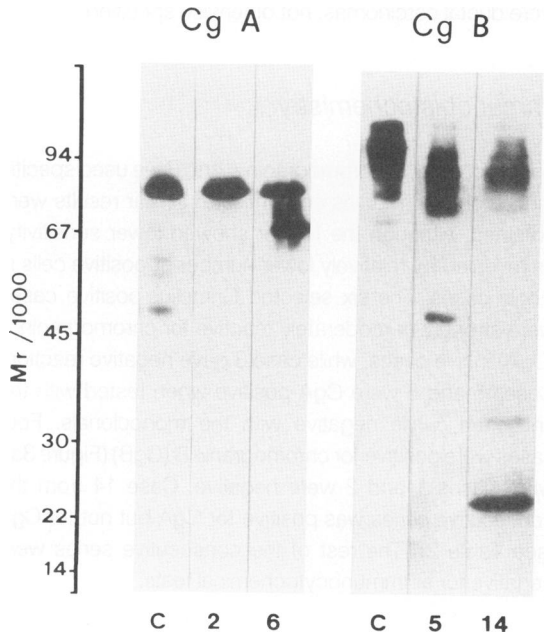

Figure 1. *One-dimensional immunoblotting of tumor extracts. The heat-stable proteins of neuroendocrine differentiated breast carcinomas were subjected to one-dimensional sodium dodecyl/sulphate electrophoresis, followed by immunoblotting with antisera against human chromogranin A (CgA) and synthetic chromogranin B (CgB).* CgA: *adrenal extract (1 μg protein) (C) and cases 2 and 6 (3 μg and 30 μg protein, respectively).* CgB: *adrenal extract (5 μg protein) (C) and cases 5 and 14 (300 μg protein both cases).*

Immunoblotting for chromogranin B was performed with two different antisera (one against human CgB and the other against a synthetic CgB peptide) with similar results. Two cases had immunoreactive bands corresponding to that obtained with adrenal CgB (Figure 1). The other 12 cases were negative. Case 1 was not tested.

No immunoreactivity for secretogranin II was found (Table 2).

Hybridization Analysis

Chromogranin A and/or B mRNA expression was clearly detected in all six NE breast carcinomas investigated by Northern blotting (Figure 2).

In one case, (number 4), both CgA and B mRNA were expressed. In three cases, (numbers 1, 2, and 14), only CgA mRNA was detectable. Two cases (numbers 3 and 5) had CgB but not CgA mRNA. All the other breast carcinomas were completely negative for CgA and B mRNA expression (Table 2).

In situ hybridization (ISH) for CgA and CgB was performed on cryostat sections of three cases: two of them

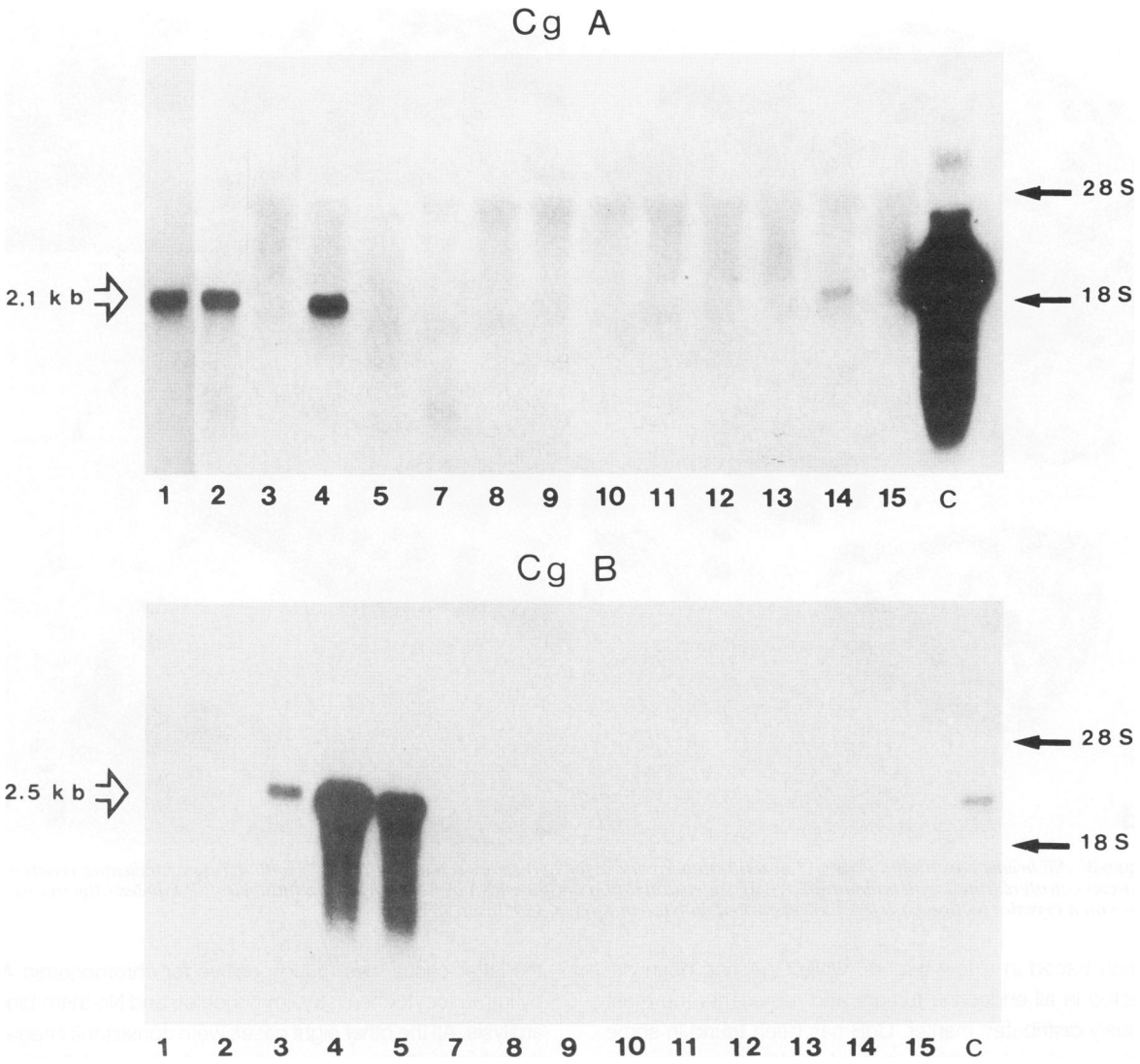

Figure 2. *Demonstration of chromogranin A (CgA) and B (CgB) mRNA in neuroendocrine breast carcinomas by Northern blot analysis using, respectively, ^{32}P-labeled antisense RNA probes and ^{32}P-labeled oligonucleotides. Lane numbers (1–15, 10 mg total RNA each) correspond to case numbers of Table 2: nos. 1, 2, 3, 4, 5, 14 NE breast carcinomas, nos. 7, 8, 9, 10, 11, 12, 13, 15 breast carcinomas with no evidence of NE differentiation. Lanes labeled C (10 mg total RNA each) represent positive controls (bovine adrenal gland for CgA and human bronchial carcinoid for CgB). CgA and CgB mRNAs migrate with the 2.1 kb, and 2.5 kb RNA classes respectively. A weak non-specific hybridization of CgA probes to ribosomal RNA was observed. Under stringent conditions no nonspecific bands were detected with CgB oligonucleotides.*

were NE breast carcinomas (numbers 3 and 4) and one case (number 10) was a breast ductal carcinoma not otherwise specified. A strong positive autoradiographic signal for CgB mRNA was observed in cases 3 and 4 (Figure 3b); differences in reaction intensity in individual cells and in different cell groups were observed. In the same tumors CgA mRNA expression was weakly detected in case 4 and absent in case 3. Case 10 was negative in ISH for both CgA and CgB.

Discussion

The present study establishes that chromogranin A and B genes are expressed in a group of breast carcinomas, confirming and extending previous immunocytochemical studies.[8,9,11]

Chromogranins are specific NE markers belonging to a family of acidic proteins originally identified in the chromaffin granules of the adrenal medulla,[37] and later shown to be widely distributed in endocrine tissues and brain.[19,38] These same proteins have also been identified by immunocytochemistry and immunoblotting, in pheochromocytomas and in various endocrine tumors (carcinoids, pancreatic endocrine tumors, thyroid medullary carcinomas, and oat cell carcinomas).[23,39,40] CgA, CgB, and secretogranin II (chromogranin C) are not always coexpressed in the same tumor: secretogranin II has only

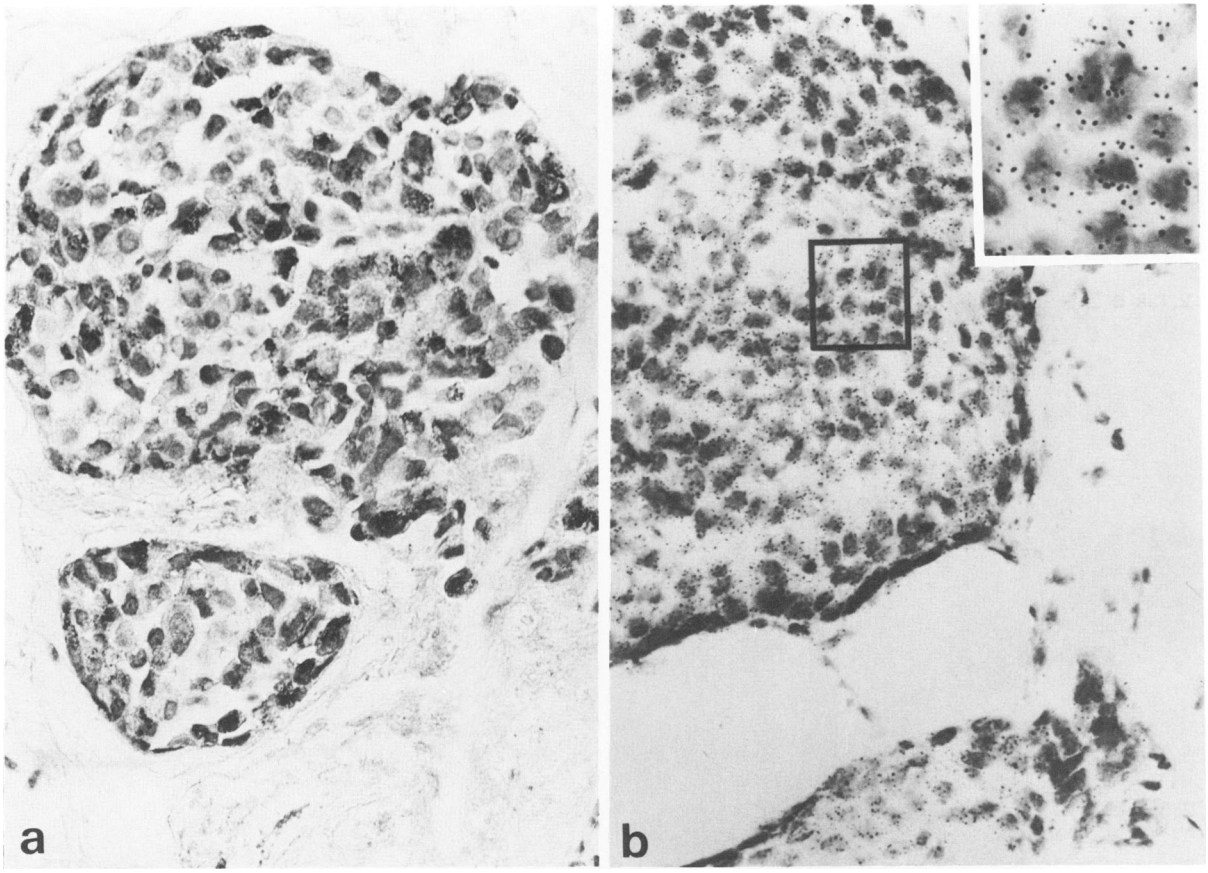

Figure 3. *NE breast carcinoma (case 4):* **a)** *immunoperoxidase for CgB shows a few positive cells with diffuse cytoplasmic reaction (nuclei counterstained with haemalum, ×350);* **b)** *visualization of CgB mRNA by* in situ *hybridization with ^{32}P-labeled oligonucleotides on a cryostat section (nuclei counterstained with haemalum, ×350; inset: ×880).*

been traced in a few tumors, while CgA has been detected in all endocrine tumors and represents the most widely distributed marker. CgB has been found in some tumors only; interestingly, it was found in all cases of benign pheochromocytomas, but less consistently in the malignant forms.[39]

Our correlated immunocytochemical, immunoblot, and Northern blot analysis of the same tumor for both CgA and CgB represents an original approach to the study of NE differentiation and of tumors in general.

Seven of the breast carcinomas of the present series were argyrophilic by the Grimelius procedure; all these tumors were immunocytochemically positive for chromogranin A and/or B and most were positive for synaptophysin, another NE marker linked to the presence of synaptic vesicles (data not shown). These tumors therefore show histochemical evidence of NE differentiation which, in a large series of breast cancers, was found expressed in 5% to 8% of cases.[11] In the present series of 15 cases, six were selected on the basis of their recognized positivity for NE markers, and nine other cases were consecutively collected from the pathology service. Only one of the latter cases was found positive for chromogranin A by immunocytochemistry, immunoblot, and Northern blot analysis. All the other eight cases were consistently negative by all methods, thus confirming the lack of NE differentiation in the vast majority of breast carcinomas.

For the immunocytochemical detection of chromogranin A we used two monoclonals and an antiserum of proved specificity[23] (the latter was, in fact, the same reagent used for the immunoblot). Interestingly, the monoclonals were negative in two cases (numbers 5 and 6), which proved reactive with the antiserum both in immunocytochemistry and immunoblot. This finding might possibly be explained by shedding of a determinant either related to neoplastic transformation or, more likely, to antigen cleavage related to peptidase activation. In addition, it has been established[41] that chromogranin detection by immunocytochemistry critically depends on fixation and embedding procedures.

Four cases of argyrophilic carcinomas were positive by immunoblotting for chromogranin A and two for chromogranin B; all cases were negative for secretogranin II. Previous studies with the same procedure in malignant

pheochromocytomas, medullary thyroid carcinomas, and in oat cell carcinomas of the lung[23,39,42] showed production of CgA and less consistently of CgB.

Hybridization demonstrated the presence of CgA mRNA in four cases and of CgB mRNA in three. Northern blots and *in situ* hybridization were performed with probes of proved specificity that recognize sequences of 2.1 Kb (CgA) and 2.5 Kb (CgB). The migration properties in our cases correspond to those reported in the literature in other organs and tumors.[30–32,43–45] In our experience the probes were found to hybridize in endocrine tissues and tumors by *in situ* hybridization and Northern blot analysis (unpublished data). In a single case of colon adenocarcinoma CgA mRNA was detected, a finding that might fit with the results of Helman et al,[46] who recently reported CgA mRNA expression in 2 of 13 cases of colon carcinomas. However, our case was found to be a signet ring cell carcinoma with NE differentiation.

The different procedures used in our study were always negative in eight of the control cases and were positive only in breast carcinomas showing NE differentiation. However, some discrepancies were observed in single cases between the results obtained from immunocytochemistry, immunoblotting, and Northern blotting, and especially between the two former. The more obvious interpretation, ie, lower specificity of the immunocytochemical procedure, was disproved by the Northern blot analysis, which established the presence of CgB mRNA in cases in which this peptide was localized by immunocytochemistry (but not immunoblotting).

The major factors that might be responsible for negative results with a single technique are related to sensitivity of the method, to storage of the product, and to autolytic phenomena. Heterogeneous distribution of NE differentiation frequently occurs in breast carcinomas.[8] Although care was taken to use adjacent tissue blocks, we cannot dismiss the possibility that heterogeneity alone might have accounted for some of the (minor) discrepancies observed.

Our study demonstrates that CgA and CgB gene expression, establishing NE differentiation, can be revealed in breast carcinomas at both mRNA and protein level.

The significance of NE differentiation in breast carcinomas is still being debated, while on the other hand, the function of peptides of the chromogranin family is still unknown.[47,48] A recent investigation has shown that NE differentiation in breast cancers is associated with expression of high levels of somatostatin receptors,[49] a finding of potential therapeutic and diagnostic[50] interest. It is well known that carcinomas of the lung with NE features have different clinical characteristics, including responsiveness to chemotherapy.[51] The identification of NE breast carcinomas by various procedures, especially by testing chromogranin(s) production at tissue level and possibly its release in the serum by radioimmunologic assay (as already demonstrated in other NE tumors),[52,53] appears to be of both biologic and clinical interest.

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