15-Prostaglandin Dehydrogenase Expression Alone or in Combination with ACSM1 Defines a Subgroup of the Apocrine Molecular Subtype of Breast Carcinoma*

Julio E. Celis‡§¶, Pavel Gromov‡§, Teresa Cabezon‡§, Jose M. A. Moreira‡§, Esbern Friis§¶, Karin Jirström**, Antonio Llombart-Bosch‡‡, Vera Timmermans-Wielenga§ §§, Fritz Rank§ §§, and Irina Gromova‡§

Established histopathological criteria divide invasive breast carcinomas into defined groups. Ductal of no specific type and lobular are the two major subtypes accounting for around 75 and 15% of all cases, respectively. The remaining 10% include rarer types such as tubular, cribriform, mucinous, papillary, medullary, and apocrine breast carcinomas. Molecular profiling technologies, on the other hand, subdivide breast tumors into five subtypes, basal-like, luminal A, luminal B, normal breast tissue-like, and ERBB2-positive, that have different prognostic characteristics. An additional subclass termed “molecular apocrine” has recently been described, but these lesions did not exhibit all the histopathological features of classical invasive apocrine carcinomas (IACs). IACs make up 0.5–3% of the invasive ductal carcinomas, and despite the fact that they are morphologically distinct from other breast lesions, there are presently no standard molecular criteria available for their diagnosis and as a result no precise information as to their prognosis. Toward this goal our laboratories have embarked in a systematic proteomics endeavor aimed at identifying biomarkers that may characterize and subtype these lesions as well as targets that may lead to the development of novel targeted therapies and chemoprevention strategies. By comparing the protein expression profiles of apocrine macrocysts and non-malignant breast epithelial tissue we have previously reported the identification of a few proteins that are specifically expressed by benign apocrine lesions as well as by the few IACs that were available to us at the time. Here we reiterate our strategy to reveal apocrine cell markers and present novel data, based on the analysis of a considerably larger number of samples, establishing that IACs correspond to a distinct molecular subtype of breast carcinomas characterized by the expression of 15-prostaglandin dehydrogenase alone or in combination with a novel form of acyl-CoA synthetase medium-chain family member 1 (ACSM1). Moreover we show that 15-prostaglandin dehydrogenase is not expressed by other breast cancer types as determined by gel-based proteomics and immunohistochemistry analysis and that antibodies against this protein can identify IACs in an unbiased manner in a large breast cancer tissue microarray making them potentially useful as a diagnostic aid.

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Breast cancer is the leading cause of cancer deaths in women today and the most common cancer among women in the Western world (1). According to the World Health Organization more than 1.2 million people will be diagnosed with breast cancer this year worldwide, and the global incidence rates are increasing. In Denmark, ~4,200 women were diagnosed with the disease in 2005; this means that the number of new cases diagnosed every year has tripled in the past 40 years. As a result, the lifetime risk of a woman developing breast cancer has increased from 1 in 28 just 50 years ago to 1 in 8 today. The 5-year survival rate is about 97% when breast cancer is detected at a more curable stage, indicating that early detection is crucial both for treatment intervention and for improving survival (2).

Breast cancer is a heterogeneous disease that encompasses a wide range of histopathological types. Established histopathological criteria classify invasive breast cancer into defined groups: two major subtypes, ductal of no specific...
type (NST)\(^1\) and lobular accounting for around 75 and 15% of all cases, respectively, in addition to rare special types, e.g., tubular, cribriform, mucinous, medullary, metaplastic, and apocrine breast carcinomas (3). However, molecular profiling technologies subdivide breast cancer tumors into five clinically relevant clusters with different prognostic characteristics. These include two estrogen receptor \(\alpha\) (ER\(\alpha\))-positive groups (luminal A and B) and three ER\(\alpha\)-negative groups comprising basal-like, normal breast tissue-like, and ERBB2/Her2-positive lesions (4, 5). This classification, however, is by no means exhaustive as work by Farmer et al. (6) recently identified a novel subset of breast tumors with increased androgen signaling and an apocrine expression profile that they termed “molecular apocrine” given that these lesions do not exhibit all the histopathological features of classical apocrine carcinomas. Molecular apocrine carcinomas encompass tumors that share some common expression characteristics with the ERBB2 class (ER\(\alpha\)/PgR/ERBB2\(^+\)) in the Stanford classification as well as with some lesions that exhibit morphological features of the triple negative group (high grade lesions; ER\(\alpha\)/PgR/ERBB2\(^+\)).

Invasive apocrine carcinomas (IACs) as defined by morphological features correspond to 0.5–3% of all invasive ductal carcinomas (IDCs) (7–10), and despite the fact that they are histologically distinct from other breast lesions there are currently no standard molecular criteria available for their diagnosis and no precise information as to their prognosis (Refs. 10–14 and references therein). IACs are generally considered as a variant of IDC (8, 10, 15), although Japaze et al. (11) recently defined separate histopathological criteria for the diagnosis of pure IACs, which have a less aggressive behavior than IDC-NST. The identification of apocrine carcinomas is further complicated by the fact that some of the less differentiated carcinomas may actually represent apocrine carcinomas that have lost their apocrine morphological characteristics, making it difficult to distinguish them from IDC-NST using purely morphological criteria (16, 17). As a result, it is likely that IACs are more frequent than previously estimated.

IACs have not been characterized at the molecular level but are generally accepted to be ER\(\alpha\)-, progesterone receptor (PgR)-negative, and androgen receptor (AR)-positive (18–21) and Bcl-2-negative (19, 22), and many are Her2/neu-negative (23). Moreover some are positive for the gross cystic disease fluid protein-15 (GCDFP-15) (9, 24–26), a marker that it is, however, not specific for these lesions (16, 17, 25). p53 expression has been observed in about 50% of invasive apocrine carcinomas (19, 27), and there have been some reports addressing the characteristics of apocrine lesions using loss of heterozygosity (22, 28, 29) and comparative genome hybridization (10, 30).

Considering the lack of histological and molecular criteria to reproducibly categorize IACs and the lack of knowledge as to which is the most appropriate treatment for patients bearing these lesions, our laboratories have embarked in a systematic proteomics endeavor aimed at identifying biomarkers that may characterize and subtype these lesions to a greater detail than it is possible today and to search for targets that may lead to the development of novel targeted therapies and chemoprevention strategies. By comparing the protein expression profiles of “blue dome” apocrine cysts and non-malignant breast epithelial tissue we have identified a few markers that are expressed by benign and non-obligatory lesions as well as by some IACs (16, 17, 31). In addition, the identification of differentially expressed proteins that characterize a specific step in the progression from early benign lesions to apocrine cancer has opened a window of opportunity for designing and testing new approaches for chemoprevention (16, 17). Here we reiterate our strategy to reveal apocrine cell markers and present novel data, based on the analysis of a considerably larger number of samples, establishing that IACs correspond to a distinct molecular subtype of breast carcinomas characterized by the expression of 15-prostaglandin dehydrogenase (15-PGDH) alone or in combination with a novel form of acyl-CoA synthetase medium-chain family member 1 (ACSM1). Moreover we show that 15-PGDH is not expressed by other breast cancer types as determined by gel-based proteomics and immunohistochemistry analysis and that antibodies against this protein can identify IACs in an unbiased manner in a large breast cancer tissue microarray making them potentially useful as a diagnostic aid.

**EXPERIMENTAL PROCEDURES**

**Sample Collection and Handling**—Tissue biopsies from clinical high risk patients\(^2\) that underwent mastectomy were collected from the Pathology Department at the Copenhagen University Hospital. Samples for gel analysis were placed in liquid nitrogen and were rapidly transported to the Institute of Cancer Biology where they were stored at \(-80^\circ\text{C}\). Samples were routinely collected within a maximum of 30–45 min from the time of surgical excision. The project was approved by the Scientific and Ethical Committee of the Copenhagen and Frederiksberg municipalities (KF 01-069/03). We also obtained paraffin-embedded tissue blocks from the Depart-

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\(^1\) The abbreviations used are: NST, no specific type; ER\(\alpha\), estrogen receptor \(\alpha\); IAC, invasive apocrine carcinoma; IDC, invasive ductal carcinoma; PgR, progesterone receptor; AR, androgen receptor; IHC, immunohistochemistry; 2D, two-dimensional; GCDFP-15, gross cystic disease fluid protein-15; ACSM1, acyl-CoA synthetase medium-chain family member 1; 15-PGDH, 15-prostaglandin dehydrogenase; TMA, tissue microarray; CK, cytokeratin; AKR1A1, aido-keto reductase family 1 member A1; apo, apolipoprotein; B-FABP, brain fatty acid-binding protein; SOD, superoxide dismutase; HMG, hydroxymethylglutaryl; SCP2, steroid carrier-binding protein 2; DCTB, Danish Centre for Translational Breast Cancer Research.

\(^2\) The criteria for high risk cancer applied by the Danish Cooperative Breast Cancer Group are age below 35 years old, and/or tumor diameter of more than 20 mm, and/or histological malignancy grade 2 or 3, and/or negative estrogen and progesterone receptor status, and/or positive auxillary status.
ment of Pathology, University of Valencia and Instituto Valenciano de Oncologia, Valencia, Spain. For these patients the personal identification number is unknown. Samples used in this study are described in Table III.

Tissue Arrays—Normal human tissue arrays containing tissues from 33 different anatomic sites (MN0661) were purchased from Pantomics, Inc., San Francisco, CA. We also used a tissue microarray (TMA) containing 498 consecutive cases of primary breast cancer diagnosed at the Department of Pathology, Malmö University Hospital between 1988 and 1992. The median age at the time of diagnosis was 65 years old (range, 27–96 years), and the median follow-up time from diagnosis to recurrent disease was 106 months (range, 0–207 months) (see Table II). The patient cohort represented in this TMA is part of the Malmö Diet and Cancer Study and has been described in detail elsewhere (32). For the present study, new tissue arrays were constructed using 2 × 1.0-mm cores from 498 cases in the cohort from which donor blocks could be retrieved. Ethical permission for the Malmö Diet and Cancer Study was obtained from the Ethical Committee at Lund University (LU 51-90) (see Table IV). In addition, a TMA (BRC1502) containing 68 human breast carcinomas present in duplicate was purchased from Pantomics, Inc.

Two-dimensional Gel Electrophoresis and Western Immunoblotting—Two dimensional polyacrylamide gel electrophoresis (IEF) was performed as described previously (33, 34). Twenty to 30 8-μm cryostat sections of frozen tissues were resuspended in 0.1 ml of lysis solution (33). The resulting lysate was frozen and kept at −20 °C until used, usually within 24–48 h (35, 36). Forty microliters were applied to the gels, and each sample was run at least in duplicate. On average three experimental replicates were performed per sample. There is little protein degradation using this procedure as determined by 2D PAGE Western blotting of a few selected samples probed with a variety of antibodies. The first and last sections of each sample were used for immunofluorescence analysis using cytokeratin 19 (CK19) antibodies as this epithelial marker is ubiquitously expressed by mammary epithelial cells (37). The availability of these pictures greatly facilitated the interpretation of the gel data as it gave a rough estimate of the ratio of glands/tumor cells to stromal tissue.

2D gels were analyzed using the PDQuest software from Bio-Rad (version 8.0.1). Silver staining, compatible with mass spectrometry, was performed according to published procedures (38). 2D gel Western immunoblotting was performed as described previously (39).
Proteins up-regulated in apocrine cysts as compared with non-malignant breast epithelia

| Protein name | Accession number (Swiss-Prot) | UniGene | M_i (×10^3)/pI | Score | Coverage | Number of recognized peptides |
|--------------|--------------------------------|---------|----------------|-------|----------|-------------------------------|
| 15-Hydroxyprostaglandin dehydrogenase (NAD^+)(PGDH) | P15428 | PGDH1 | 29/5.6 | 212 | 45 | 12/13 |
| 3-Hydroxyisobutyryl-coenzyme A hydrolase isoform 1 | Q6NVV1 | HIBCH | 44/8.4 | 227 | 40 | 15/16 |
| Acyl-CoA dehydrogenase, medium chain | P11310 | ACADM | 47/8.6 | 224 | 33 | 15/18 |
| Acyl-CoA synthetase medium-chain family member 1 | Q08AH1 | ACSM1 | 66/8.4 | 253 | 29 | 19/22 |
| Acyl-CoA binding protein | P07108 | DBI | 10/6.1 | 143 | 83 | 9/14 |
| Aldehyde dehydrogenase 4A1, mitochondrial | P30338 | ALDH4A1 | 62/8.2 | 254 | 38 | 19/25 |
| Aldo-keto reductase family 1 member A1 | P14550 | ALDR1 | 36.8/6.3 | 254 | 44 | 15/22 |
| Aldo-keto reductase family 1 member C2 | P52895 | AKR1C2 | 37/7.1 | 191 | 37 | 14/20 |
| Aldose reductase-like 1 (novel isoform) | gi|122937516 | 39.7/6.0 | 210 | 50 | 18/24 |
| Apolipoprotein D | P05090 | APOD | 21.6/5.06 | 195 | 47 | 13/21 |
| Apolipoprotein J | P01099 | SERPINA1 | 47/5.4 | 186 | 25 | 14/19 |
| Arachidonate 15-lipoxygenase type II | O15296 | ALOX15B | 76.6/5.8 | 210 | 27 | 15/17 |
| (without N-terminal end) | | | | | | |
| Aspartate aminotransferase | P17174 | GOT1 | 46.4/6.6 | 147 | 23 | 10/13 |
| β-Glucuronidase | P08236 | GUSB | 69/6.5 | 269 | 26 | 22/28 |
| Catechol O-methyltransferase | P21964 | COMT | 30.5/5.3 | 206 | 49 | 10/10 |
| Cathepsin D | P07339 | CTSD | 38/5.6 | 220 | 40 | 17/18 |
| CCG1-interacting factor B | Q96IU4 | ABHD14B | 22.5/9 | 163 | 60 | 9/12 |
| Fatty acid synthase | P49327 | FASN | 276/6.6 | 124 | 8 | 20/27 |
| Fatty acid binding-protein, brain | O15540 | FABP7 | 14.8/5.4 | 166 | 65 | 10/12 |
| Flavin reductase | P30043 | BLVRB | 22/7.3 | 200 | 74 | 11/15 |
| Glutathione S-transferase Theta 2 | P30712 | GSTT2 | 27.5/6.01 | 186 | 41 | 11/12 |
| HMG-CoA synthase | P54868 | HMGC52 | 57.8/4.8 | 146 | 16 | 13/17 |
| Isocitrate dehydrogenase (NADP) cytoplasmic | Q75874 | IDH1 | 46.9/6.5 | 253 | 44 | 18/21 |
| Isovaleryl-diphosphate Δ-isomerase 1 | Q13907 | ID1 | 26.7/5.9 | 240 | 54 | 12/12 |
| Isovaleryl-CoA dehydrogenase | P26440 | IVD | 47/8.4 | 197 | 27 | 14/19 |
| Membrane-associated progesterone receptor component 1 | O00264 | PGRMC1 | 22/4.5 | 227 | 37 | 11/11 |
| Methylcrotonoyl-CoA carboxylase β chain | Q9HCC0 | MCCC2 | 58/7.6 | 346 | 46 | 24/25 |
| Nicotinate-nucleotide pyrophosphorylase | Q15274 | OQRT | 31/5.8 | 151 | 40 | 11/20 |
| Nucleosomin | P06748 | NPM1 | 33/4.6 | 140 | 39 | 11/14 |
| Phosphoenoopyruvate carboxykinase, mitochondrial | Q16822 | PCK2 | 71.6/7.5 | 230 | 24 | 17/20 |
| Prolactin-inducible protein | P12273 | GCDFP15 | 16.9/8.2 | 188 | 67 | 11/18 |
| Sterol carrier protein 2 | P22307 | SCP2 | 59.8/6.4 | 163 | 25 | 13/17 |
| Superoxide dismutase (Cu/Zn) | P00441 | SOD1 | 16/5.7 | 168 | 51 | 9/13 |
| Transketolase | P29401 | TKT | 68/7.6 | 272 | 34 | 19/20 |

a Up-regulated proteins were determined by the analysis of silver-stained 2D gels. The raw-spectra data is given in Supplemental Table 1.

The second number corresponds to the total numbers of peptides subjected to the analysis.

Protein Identification by Mass Spectrometry—After scanning, the gels were placed in 7.5% acetic acid, 50% ethanol, 0.05% formalin for 1 h; washed 3 × 30 min in 7.5% acetic acid, 10% ethanol; and stained with silver nitrate according to procedures compatible with mass spectrometry (40). Protein spots were excised from dry gels, and the gel pieces were rehydrated in water. Gel pieces were detached from the cellophane film and cut into 1-mm² pieces followed by “in-gel” digestion as described previously (41). Samples were prepared for analysis by applying 2 μl of digested and extracted peptides on the surface of a 400/384 AnchorChip target (Bruker Daltonics, GmbH) followed by co-crystallization with α-cyano matrix. Mass spectrometry was performed using a Reflex IV MALDI-TOF mass spectrometer equipped with a Scout 384 ion source. All spectra were obtained in positive reflector mode with delayed extraction using an acceleration voltage of 28 kV. The resulting mass spectra were internally calibrated using the autodigested tryptic mass values visible in all the spectra. Calibrated spectra were processed by the Xmass 5.1.1 and BioTools 2.1 software packages (Bruker Daltonics, GmbH). Irrelevant masses (matrix, metal adducts, and autodigested tryptic masses as well as masses of tryptic peptides from keratins) were excluded from the analysis by manual examination of all spectra by pairwise comparison. The spectrum of interest was superimposed with the spectrum obtained from the negative control (set of peptides from the empty matrix, metal adducts, and autodigested tryptic masses as well as masses of tryptic peptides from keratins).
NCBI Ntr (version, May 15, 2008; 685,787 mammalian entries) database using the MASCOT search engine (version 2.2, released January 28, 2008, Matrix Science Ltd.). No restriction on the protein molecular mass and taxonomy was applied. A number of fixed (acrylamide-modified cysteine, i.e. propionamide/carbamidomethylation) and variable modifications (methionine oxidation and protein N terminus acetylation) were included in the search parameters. The peptide tolerance did not exceed 50 ppm, and a maximum of one trypsin missed cleavage was allowed. Protein identifications were considered to be confident when the protein score of the hit exceeded the threshold significance score of 70 ($p / H11021_0.05$) and no less than six peptides were recognized. Often the peptides identified matched equally well to multiple database entries using the NCBI Ntr database, which is why the second/final search was performed using the same parameters but the UniProtKB/Swiss-Prot 55.3 (19,372 human entries) database, and if the number and the sequence of the recognized peptides were identical to the first search, the Swiss-Prot accession number was assigned for the identified protein. In a few rare cases with a low allowed $p$ value a spot identity was confirmed by Western blotting.

**Antibodies**—Anti-peptide antibodies against ACSM1 were prepared by Eurogentec (Liege, Belgium). The rabbit polyclonal antibody raised against 15-PGDH has been described previously (16, 17, 42). Monoclonal antibodies against p53 (clone D0-7), ERα (clone 1D5), PgR (clone PgR636), AR (clone AR441), and Bcl-2 (clone 124) were purchased from DakoCytomation (Glostrup, Denmark). The antibody against CK19 (clone A53-B/A2.26) was from NeoMarkers (Lab Vision Corp., Fremont, CA). The rabbit anti-peptide antibody against human CK15 was purchased from Aviva Systems Biology. The monoclonal antibody against GATA-3 was obtained from Santa Cruz Biotechnology, Inc. The specificity of most of the antibodies used in this work was determined by 2D PAGE immunoblotting using a number of tissues and cell lines representing positive and negative controls (36).

**Immunohistochemistry (IHC)**—Following surgery, fresh tissue blocks were immediately placed in formalin fixative and paraffin-embedded for archival use. Six-micrometer sections were cut from the tissue blocks and mounted on Super Frost Plus slides (Menzel-Gläser, Braunschweig, Germany), baked at 60 °C for 60 min, deparaffinized, and rehydrated through graded alcohol rinses (43). Heat-induced antigen retrieval was performed by immersing slides in 10 mM citrate buffer (pH 6.0) and microwaving in a 750-watt microwave oven for 10 min. The slides were then cooled at room temperature for 20 min and rinsed abundantly in tap water. Nonspecific staining of slides was blocked (10% normal goat serum in PBS buffer) for 15 min, and endogenous peroxidase activity was quenched using 0.3% H2O2 in methanol for 30 min. Antigen was detected with a relevant primary antibody followed by a suitable secondary antibody conjugated to a peroxidase complex (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody; DakoCytomation). Finally color development was done with 3,3′-diaminobenzidine (Pierce) as a chromogen to detect bound antibody complex. Slides were counterstained with hematoxylin. Standardization of the dilution, incubation, and development times appropriate for each antibody allowed an accurate comparison of expression levels in all cases. At least three independent stainings of the samples were performed.
for each antibody. Sections were imaged using either a standard bright field microscope (Leica DMRB) equipped with a high resolution digital camera (Leica DC500) or a motorized digital microscope (Leica DM6000B) controlled by Surveyor software (Objective Imaging Ltd., Cambridge, UK) for automated scanning and imaging that enables tiled mosaic image creation. Original magnification for all images is 200x.

### RESULTS

#### Identification of Apocrine Cell Markers

To search for biomarkers specific to the apocrine phenotype we carried out a comparison of the protein expression profiles generated by 2D gel-based analysis of four pairs of blue dome apocrine cysts (16, 44) and non-malignant breast epithelia followed by protein identification using mass spectrometry, antibody preparation, and validation of results on archival paraffin-embedded tissue by IHC (16, 17). Blue dome cysts are composed of cells that have undergone apocrine metaplasia, i.e. transformed from breast epithelial cells into an apocrine sweat gland type of cells, and are surrounded by a layer of myoepithelial cells that line the basal membrane (Fig. 1A; compare with normal epithelial gland in Fig. 1C). Fig. 1, B and D, show silver nitrate-stained IEF 2D gels of whole protein extracts from apocrine cysts and non-malignant breast epithelia depicting some of the proteins that are expressed at a much higher level by the apocrine cells as judged by PDQuest analysis. These include, to name a few, 15-PGDH, a novel form of ACSM1, aldo-keto reductase family 1 member A1 (AKR1A1), apolipoprotein D (apoD), apoJ, β-glucuronidase, brain fatty acid-binding protein (B-FABP), cathepsin D, CuZn-superoxide dismutase (CuZn-SOD), flavin reductase, GCDFP-15, hydroxymethylglutaryl (HMG)-CoA synthase, and steroid carrier-binding protein 2 (SCP2) (Fig. 1B) (16). These and other proteins up-regulated in apocrine cysts are listed in Table I.

**Table II**

| Tissue-anatomic site | Histology | 15-PGDH | ACSM1 |
|----------------------|-----------|---------|-------|
| Adrenal gland | Normal | Negative | Negative |
| Bladder | Normal | Positive (umbrella cells) | Negative |
| Bone marrow | Normal | Negative | Negative |
| Eye | Normal | Negative | Negative |
| Breast | Normal | Negative | Negative |
| Cerebellum | Normal | Negative | Negative |
| Cerebral cortex | Normal | Negative | Negative |
| Fallopian tube | Normal | Negative | Negative |
| Esophagus | Normal | Negative | Negative |
| Stomach | Normal | Positive weak (gastric glands) | Negative |
| Small intestine | Normal | Negative | Negative |
| Colon | Normal | Negative | Negative |
| Rectum | Normal | Negative | Negative |
| Heart | Normal | Negative | Positive (cardiac muscle) |
| Kidney | Normal | Positive weak (proximal convoluted tubes) | Negative |
| Liver | Normal | Negative | Negative |
| Lung | Normal | Negative | Negative |
| Ovary | Normal | Negative | Negative |
| Pancreas | Normal | Negative | Negative |
| Parathyroid | Adenoma | Negative | Negative |
| Pituitary gland | Normal | Negative | Negative |
| Placenta | Normal | Negative | Negative |
| Prostate | Normal | Negative | Negative |
| Skin | Normal | Negative | Negative |
| Spinal cord | Normal | Negative | Negative |
| Spleen | Normal | Negative | Negative |
| Striated muscle | Normal | Negative | Negative |
| Testis | Normal | Negative | Negative |
| Thymus | Normal | Negative | Negative |
| Thyroid | Normal | Negative | Negative |
| Tonsil | Normal | Negative | Negative |
| Uterus-cervix | Normal | Negative | Negative |
| Uterus-endometrium | Normal | Negative | Negative |

a TMA containing 33 types of normal human tissues (MNO661; Pantomics, Inc.) in duplicates. The TMAs were used 1 week after shipment.
b The antibody was used at a dilution of 1:3,000.
c The antibody was used at a dilution of 1:400.
d The tissues were from autopsies.
e Only a handful of cells of unknown origin stained.
Expression of 15-PGDH and ACSM1 by Benign Apocrine Lesions and Normal Human Tissues

Apocrine differentiation of breast epithelial cells is observed in a spectrum of benign lesions of the breast and is usually found in apocrine cysts, apocrine hyperplasia within papilomas, and apocrine changes in sclerosing adenosis (Refs. 10, 13, and 31 and references therein). Fig. 2 shows representative examples of IHC stainings of apocrine microcysts (Fig. 2, A and B), an intraductal papilloma with apocrine differentiation (Fig. 2, C and D), and apocrine adenosis (Fig. 2, E and F) with our 15-PGDH and ACSM1 rabbit polyclonal antibodies, respectively. These samples are part of a large sample collection described previously (16, 31). As expected, all these apocrine lesions were ERα−, PgR−, and AR+ (results not shown; Refs. 19–21). Although most of the apocrine cysts analyzed so far immunoreacted with both the 15-PGDH and ACSM1 antibodies (illustrated in Fig. 2, A and B), we encountered some ERα−, PgR−, AR+ apocrine cysts present in “normal biopsies” that stained only with the 15-PGDH antibody as determined by IHC analysis of serial sections (Fig. 2, G and H). Interestingly, human apocrine sweat glands obtained from the skin in the axilla also stained preferentially with the 15-PGDH antibody as illustrated in Fig. 2, I and J, implying that there may be more than one type of apocrine cysts. The specificity of both antibodies was confirmed by 2D PAGE Western immunoblotting as shown in Fig. 2, K and L.

IHC analysis of sections of non-malignant tissue biopsies collected from about 100 patients with invasive breast cancer (described in detail in previous publications (16, 17, 31) showed that the two apocrine cell markers are not expressed by cells present in terminal ductal lobular units, type II flat microcysts, stroma, or any other cell type present in the mammary gland (see Fig. 2), underscoring their value as markers for apocrine lesions. Both biomarkers are far superior for discriminating apocrine cells as compared with GCDFP-15 (results not shown), a protein generally regarded as a specific functional marker of apocrine cells (9, 24, 25).

The expression pattern of the two biomarkers in pathologically normal human tissues was determined by IHC using TMAs containing 33 types of normal human tissues present in duplicate; the tissue panel present in this TMA includes the samples recommended by the Food and Drug Administration in its guidelines for testing of cross-reactivity during the development of an immunostaining protocol. As noted in Table II, both the 15-PGDH and ACSM1 antibodies displayed restricted immunoreactivity with only very few tissues showing significant positive staining (Fig. 3, Panel A–F). For 15-PGDH, the highest immunoreactivity was observed in the umbrella cells of the transitional epithelium of the urinary bladder (Fig. 3A) with less intense staining being observed in stomach (gastric glands) and kidney (proximal convoluted tubes) (results not shown). It should be


### Table III

**Immunophenotype of IACs**

| Tumor | Age | Type | Size | Grade | Her2/neu status | ALN | ERα, PgR, AR | 15-PGDH | ACSM1 | Bcl-2 | GATA-3 | p53 | CK15 |
|-------|-----|------|------|-------|----------------|-----|--------------|---------|-------|-------|--------|-----|------|
| 1     | 67  | D    | 16   | 2     | 2+             | 1.67| Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 2     | 79  | D    | 24   | 3     | 2+             | 1.7 | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 3     | 58  | D    | 12   | 2     | 1+             | N− | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 4     | 54  | D    | 30   | 2     | 1.33           | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 5     | 44  | D    | 21   | 3     | N−             | 1.56| Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 6     | 79  | D    | 30   | 3     | 0              | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 7     | 41  | D    | 30   | 3     | 3+             | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 8     | 83  | D    | 28   | ND   | 2+             | 1.03| Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 9     | 73  | D    | 23   | 3     | 3+             | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 10a   | 80  | D    | 16   | 3     | 2+             | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 11a   | 78  | D    | 25   | 3     | 0              | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 12a   | 61  | D    | 30   | 2     | 3+             | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 13a   | 41  | D    | 16   | 2     | 3+             | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 14a   | 54  | D    | 22   | 2     | 3+             | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 15a   | 55  | D    | 14   | 2     | ND             | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 16a   | 53  | D    | 60   | 2     | 3+             | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 17a   | 56  | D    | 35   | 1     | 1+             | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 18a   | 79  | D    | 25   | 3     | 2+             | 1.13| Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 19a   | 81  | D    | 22   | 2     | ND             | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 20a   | 49  | D    | 8    | 3     | 3+             | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |
| 21a   | 57  | D    | 19   | 2     | 3+             | N+ | Pos          | Pos     | Pos   | Pos   | Pos    |     |      |

*In years.

- D, ductal carcinoma.

- The tumor size is given in mm.

- Her2 status was defined as positive or amplified respectively, either as IHC level of 3+ (HERCEPTEST), or as FISH ratio of 2.0 or higher (DAKO kit). IHC level 2+ was defined as equivocal and followed by FISH (56).

- ALN, axillary lymph node. N+, metastasis in lymph nodes; N−, no metastasis detected in lymph nodes (see also “Experimental Procedures”).

- ERα, estrogen receptor alpha; PgR, progesterone receptor; AR, androgen receptor. ERα and PgR status was determined by IHC analysis according to the Danish Breast Cancer Cooperative Group (DBCG) guidelines. Tumors were regarded as negative when receptors were expressed in less than 10% of tumor cell nuclei.

- Pos, positive; Neg, Negative.

- From the Department of Pathology, Copenhagen University Hospital.

- From the Department of Pathology, University of Valencia and Instituto Valenciano de Oncologia.

- ND, not determined.

Stressed that negative staining does not necessary mean absence of the marker as the antibody may not be sensitive enough to detect low levels of the protein. Examples of negative staining are presented in Fig. 2, B (colon) and C (skin). In the case of ACSM1, the antibody immunoreacted mainly with heart muscle tissue (Fig. 3D and Table II) and with some cells present in the vasculature (Fig. 3E). Examples of staining of bladder and esophagus tissue sections are presented in Fig. 3, E and F, respectively. 2D PAGE (Fig. 3, Panel B) and IHC (not shown) analysis of independent samples of human bladder urothelium (Fig. 3G), colon (Fig. 3H), and skin (Fig. 3I) available in our tissue bank collection established that immunostaining with the 15-PGDH antibody accurately reflected absolute levels of protein expression. Because of the low levels of ACSM1 in apocrine cells (Fig. 1B) and the fact that this protein migrates in a very densely populated area of the 2D gels we have so far been unable to verify a correlation between immunostaining and absolute levels of expression of the ACSM1 protein.

**Expression of 15-PGDH and ACSM1 by IACs**

Previous studies from our laboratory based on the analysis of a very limited number of IACs suggested that 15-PGDH might be expressed preferentially by these carcinomas (16, 17). To
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A total of 21 ERα−, PgR−, and AR+ IACs diagnosed at the Pathology Departments at the Copenhagen University Hospital and the University of Valencia and Instituto Valenciano de Oncologia were analyzed by IHC using the 15-PGDH and ACSM1 antibodies. In eight cases (Copenhagen University Hospital) we also had access to snap frozen tissue biopsies, and these were used for 2D PAGE analysis in triplicate.

Of the 21 carcinomas analyzed by IHC, six had invasive cells with the immunophenotype 15-PGDH/ACSM11+/+, eight had invasive cells with the immunophenotype 15-PGDH/ACSM11−/−, and seven had invasive cells with the immunophenotype 15-PGDH/ACSM11+/− (Table III). Additional immunophenotyping of the 21 carcinomas showed that with two exceptions (tumors 14 and 19), all were Bcl-2 and GATA-3 negative and several were p53 positive (Table III). There is compelling evidence indicating that benign apocrine proliferations are Bcl-2 negative (19, 22); GATA-3 is a factor involved in luminal epithelial cell differentiation (46, 47). Tumor 14 (15-PGDH/ACSM11+/−) contained cells that were GATA-3 positive and negative and it may correspond to a mix lesion. Tumor 19 (15-PGDH/ACSM11+/−), on the other hand, contained predominantly Bcl-2 positive cells and may not correspond to an IAC (Table III). The other six 15-PGDH/ACSM11−/− lesions might correspond to IACs that lost their ability to express the two markers as a result of tumor progression (Table III).

IHC examples of 15-PGDH/ACSM11+/+, 15-PGDH/ACSM11+/−, and 15-PGDH/ACSM11−/− lesions are given in Fig. 4, A and B (tumor 1), D and E (tumor 10), and G and H (tumor 16). Relevant areas of the 2D gels of the corresponding tumors are presented in Fig. 4, C, F, and I, respectively. The expression of ACSM1 could not be confirmed by 2D PAGE as the protein migrates in a very crowded area of the gels. However, its presence was confirmed in tumor 3 by 2D gel Western immunoblotting (Fig. 2L).

For reference, Fig. 5 shows full silver-stained 2D gel images of a 15-PGDH/ACSM11−/− IAC (Fig. 5A; tumor 3) and a ductal (Fig. 5B) and a lobular (Fig. 5C) invasive carcinoma as well as a mucinous lesion (Fig. 5D). As shown, none of the non-apocrine carcinomas express 15-PGDH at least at the level of detection provided by silver staining, a fact that has been confirmed by similar analysis of about 150 breast carcinomas collected by the DCTB program as well as by IHC on an even larger number of tumor samples. Additional 2D gels of IACs analyzed in this study are presented in supplemental Fig. 2.
Antibodies—A TMA consisting of 498 consecutive cases of primary breast cancer diagnosed between 1988 and 1992 at the Department of Pathology, Malmö University Hospital was used to identify carcinomas that immunoreacted with the 15-PGDH and ACSM1 antibodies. The clinicopathological parameters of the carcinomas in the consecutive cohort are given in Table IV. Only four of the 498 carcinomas reacted positively with the 15-PGDH antibody, and two of these were also positive for ACSM1 (Table V). The staining of core 380 with both antibodies is shown in Fig. 6, A and B. All four carcinomas were ER−/H9251−/H11002, PgR−/H11002−, and AR−/H11001− and were originally diagnosed as IDCs (Table V). Their apocrine nature was confirmed by two of the pathologist following re-examination of the slides. That the 15-PGDH-positive IACs are rare was further confirmed by IHC analysis of an additional TMA containing 68 breast carcinomas in duplicate (see “Experimental Procedures”). Only one tumor in the array (G4, H4; Pantomics, Inc.) reacted positively and had the phenotype ER−/H9251−/H11002, PgR−/H11002−, and AR−/H11001− (Table III; results not shown for CK19) suggesting that the latter may have evolved from differentiated lineage-restricted luminal cells (phenotype CK15+/CK19+/ERα+/PgR−/AR−/Bcl-2−/GATA-3−) as a result of genetic and epigenetic changes (36). The possibility, however, that some IACs may be derived from undifferentiated breast luminal precursor cells cannot be discarded at present.

Evolution of the IAC Phenotype

Recent studies in our laboratories identified single layers of CK15+/CK19− columnar cells contiguous to cells undergoing apocrine metaplasia and suggested that apocrine metaplasia may involve reprogramming of CK15+/CK19+/ERα+/PgR+/AR+/Bcl-2+/GATA-3+ luminal cells to generate CK15−/CK19+/ERα−/PgR−/AR−/Bcl-2−/GATA-3− apocrine cells (36). IHC analysis of all the carcinomas listed in Table III using a CK15-specific antibody revealed that with one exception (tumor 1; Table III) all other carcinomas had the phenotype CK15+/CK19+/ERα+/PgR−/AR−/Bcl-2−/GATA-3− (Table III; results not shown for CK19) suggesting that the latter may have evolved from differentiated lineage-restricted luminal cells (phenotype CK15+/CK19−/ERα+/PgR−/AR+/Bcl-2−/GATA-3−) as a result of genetic and epigenetic changes (36). The possibility, however, that some IACs may be derived from undifferentiated breast luminal precursor cells cannot be discarded at present.

DISCUSSION

Considering the lack of histological and molecular criteria to reproducibly categorize invasive IACs and the need to establish the most appropriate treatment for patients bearing these lesions it is essential to identify biomarkers that can subdivide IACs to greater molecular detail and that may serve as potential targets for novel targeted therapies and chemoprevention strategies. Toward these aims, the studies presented here illustrate the value of using proteomics technologies in combination with IHC in an iterative fashion to identify a distinct subgroup of the apocrine molecular subtype of breast carcinomas as well as to generate markers for dissecting some of the stages involved in apocrine metaplasia (16, 17, 31).
From our studies it is apparent that IACs correspond to a heterogeneous but distinct subgroup of breast carcinomas. Some of these lesions may lose the expression of 15-PGDH during tumor progression (Fig. 7) and may become morphologically and expression-wise very similar to IDC (NST). The heterogeneity of apocrine carcinomas has also been pointed out in recent studies by Japaze et al. (11) who defined histopathological criteria for the diagnosis of pure invasive apocrine carcinoma, an intermediate group of apocrine tumors that represent a distinct clinicopathological entity and that have a less aggressive behavior than IDC (NST) (11). Whether these tumors share common characteristics with the 15-PGDH-positive lesions described in our studies is at present unknown and will be the subject of further studies.

Recently Farmer et al. (6) identified a subclass of basal cell breast carcinomas using gene expression microarrays that they termed molecular apocrine. These lesions, which present increased androgen signaling, share common features with the ERBB2 class in the Stanford classification (48). Molecular apocrine carcinomas are positive for HMG-CoA reductase, a protein marker that we have shown is lost prior to 15-PGDH in the course of apocrine tumor progres-
sion (16, 17), and are ERα- and PgR-negative but express AR. Other genes expressed by these lesions include growth hormone receptor, prolactin receptor, epidermal growth factor receptor, NADPH-flavin reductase, steroyl-CoA desaturase, fatty acid synthase, aldehyde dehydrogenase, E-cadherin, Rho family GTPase 1, short-chain dehydrogenase/reductase (SDR) family member 2, and peroxisomal \( \Delta^1 \)- and \( \Delta^2 \)-enoyl-CoA isomerase. Both NADPH-flavin reductase and aldehyde dehydrogenase were also identified in our studies (Table I). Interestingly the authors did not identify 15-PGDH or ACSM1 as RNAs overexpressed by the molecular apocrine lesions. Also no significant variation in the levels of 15-PGDH was observed in the seminal studies of Perou et al. (4) and Sørlie et al. (5). This is not surprising given the rarity of IACs.

Further studies will be needed to determine the relationship between the IACs positive for 15-PGDH we have described and the lesions in the molecular apocrine subtype described by Farmer et al. (6).

One of the main challenges we foresee in the future, besides revealing additional apocrine markers to further characterize IACs, will be the search for markers that are able to detect IACs that have lost important morphological features as well as known apocrine markers (Fig. 7). For example, in the study presented here two of the 15-PGDH/ACSM1 \(^{-/-}\) carcinomas, tumors 16 and 17, contained less than 1% of cells (spread throughout the various areas of the lesion) that stained with the 15-PGDH antibody suggesting that these tumors may correspond to more advanced, "pro-
pressed" IACs (results not shown). To search for these late event markers it will be necessary to compare the protein expression profiles of 15-PGDH-positive lesions with tumors for which there is convincing evidence that they are derived from IACs but have progressed toward a new phenotype losing qualifying features. By comparing their expression patterns with those of non-apocrine carcinomas it should be possible to identify specific markers that recognize these progressed apocrine lesions and to use antibodies against these markers to screen TMAs containing large number of tumors with appropriate clinical information and follow-up. Only by ensuring accurate molecular categorization of IACs can one begin to assess their true incidence and clinical outcome and consequently determine the prognosis of patients bearing IACs and the most appropriate treatment regimen. One aspect of the present study that should be strenuously emphasized is that the strategy we used for discovery of apocrine-specific biomarkers and initial validation is entirely dependent on the quality of the initial tissue samples analyzed. Given that IACs are poorly characterized, collecting a sufficient number of samples can be a daunting challenge as most pathologists do not routinely record the presence of apocrine features/areas present in lesions classified as IDCs.

Another pressing priority is the identification of specific biomarkers that can be used to detect apocrine carcinomas at an early stage as well as to reveal precancerous lesions that are likely to progress to invasive disease (16, 17, 31). These markers will permit accurate preoperative diagnosis and will complement the cytology-based analysis of cells found in the cyst fluid of patients indicated by mammography. As follows, markers such as 15-PGDH, HMG-CoA reductase, and cyclooxygenase 2 that are expressed by apocrine cells (16, 17) have already provided a window of opportunity for pharmacological intervention not only in a therapeutic manner but also in a chemopreventive setting (16, 17, 49–53).

Finally it should be mentioned that 15-PGDH was reported by Wolf et al. (54) as a tumor suppressor of human breast cancer. Although we cannot directly compare our results with the data reported by these authors given that most of their study consisted of RT-PCR-based analyses of clinical samples and Western blotting of two cell lines, we attempted to correlate their data with our observations by performing 2D PAGE analysis of some of the cell lines examined in their study (MDA-MB-231 and MCF-7; data not shown). We were unable to detect expression of 15-PGDH in any of the cell lines examined as determined by 2D gels visualized with silver staining. The observation we report here that non-malignant breast epithelial and non-apocrine tumors do not express 15-PGDH protein is based on the analysis of clinical samples using two methods, namely IHC and 2D PAGE. We cannot exclude, however, the possibility that there may be differences in the levels of expression of this protein between benign and malignant breast epithelium that are below the level of detection of the technologies we used.

In conclusion, we have presented evidence that IACs correspond to a distinct molecular subtype of breast carcinomas characterized by the expression of apocrine markers. The next step in our studies will be the establishment of a diagnostic test aimed at the identification of IACs. This implies the implementation of the use of 15-PGDH antibodies in routine pathology, which will require the preparation and validation of reagents with broad availability and immunoreactivity similar to that of the antibody used in this study.

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