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Breast carcinomas fulfill the Warburg hypothesis and provide metabolic markers of cancer prognosis

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The aim of this study was to investigate selected proteomic markers of the metabolic phenotype of breast carcinomas as prognostic markers of cancer progression. For this purpose, a series of 101 breast carcinomas and 13 uninvolved breast samples were examined for quantitative differences in protein expression of mitochondrial and glycolytic markers. The β-subunit of the mitochondrial H^+-ATP synthase (β-F1-ATPase) and heat shock protein 60 (Hsp60), and the glycolytic glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase were identified by immunological techniques. Correlations of the expression level of the protein markers and of the ratios derived from them were established with the clinicopathological information of the tumors and the follow-up data of the patients. The metabolic proteome of breast cancer specimens revealed a pronounced shift towards an enhanced glycolytic phenotype concurrent with a profound alteration on the mitochondrial β-F1-ATPase/Hsp60 ratio when compared with normal samples. Discriminant analysis using markers of the metabolic signature as predictor variables revealed a classification sensitivity of ∼97%. Kaplan–Meier survival analysis showed that several of the proteomic variables significantly correlated with overall and disease-free survival of the patients. The expression level of β-F1-ATPase *per se* allowed the identification of a subgroup of breast cancer patients with significantly worse prognosis. Multivariate Cox regression analysis indicated that tumor expression of β-F1-ATPase is a significant marker independent from clinical variables to assess the prognosis of the patients. We conclude that the alteration of the mitochondrial and glycolytic proteomes is a hallmark feature of breast cancer further providing relevant markers to aid in the prognosis of breast cancer patients.

Abbreviations: BEC, bioenergetic cellular; Hsp60, heat shock protein 60; LDH, lactate dehydrogenase; PK, pyruvate kinase; β-F1-ATPase, beta subunit of the mitochondrial H^+-ATP synthase.

Introduction

Breast cancer is the most common neoplasia among women in Western Europe and North America, with an estimated incidence of 7.6–9.1/10 000 inhabitants per year (1). A genetic background associated with germline mutations of the *BRCA1* and *BRCA2* genes is responsible for only a small fraction (5–10%) of the cases (2). Invasive ductal carcinoma accounts for 80% of these tumors, followed by lobular, tubular, medullary and other special types. Unfortunately, prognostic markers currently accepted for clinical use, such as nodal status, tumor size, histological grade, steroid receptor status (3,4) and others (5) do not adequately identify patients at higher risk of relapse. Therefore, additional prognostic markers for the clinical management of breast cancer patients are needed.

Nowadays, we are facing a renaissance of the role of mitochondria in cancer biology (6,7) and therefore, attempts to analyse the expression pattern of the genes and proteins that are associated with the mitochondrial phenotype of a particular type of tumor are likely to contribute to the diagnosis and prognosis of cancer patients (8–10). Within this context, a high frequency of somatic mitochondrial DNA (mtDNA) mutations in breast (11,12) as well as in other human cancers (13), that affect the energetic capability of the organelle have already been described. Likewise, inherited heterozygous mutations in nuclear genes that impact on the bioenergetic function of mitochondria have been shown to predispose to two different types of inherited neoplasia syndromes (14–16). More recently, the apparent conundrum of whether mtDNA mutations found in tumors are a cause or a consequence of the carcinogenic process has been partially solved after the demonstration that pathogenic mtDNA mutations that impinge on mitochondrial energy transduction do play a relevant role in the etiology of cancer by any of the following mechanisms: excessive reactive oxygen species signaling (17), diminished cellular apoptotic potential (18) or mitochondria to nucleus signaling of a cellular invasive phenotype (19). At the mitochondrial protein level, a specific repression of the expression of the β-catalytic subunit of the H^+-ATP synthase (β-F1-ATPase), a rate-limiting component of mitochondrial oxidative phosphorylation, has been documented in rat hepatocarcinomas (20) as well as in human tumor biopsies of liver, colon, kidney, lung, breast, gastric and esophageal cancer patients (21–23). These findings have been recently confirmed (24,25) and extended to other carcinomas (26). Interestingly, in these studies it was also observed that protein markers of the glycolytic pathway were concurrently up-regulated in most of the human solid tumors analysed. Therefore, we reasoned that the metabolic phenotype of the cell could provide markers for the analysis of cancer progression (21). For this purpose, we developed a straightforward proteomic-based analysis of cancer biopsies that we defined as the bioenergetic cellular index (BEC index) (21). The BEC index expresses putative

alterations on the mitochondrial proteome brought about by carcinogenesis relative to the glycolytic potential of the cell (21). Remarkably, we found that the relative expression level of the β-F1-ATPase in lung (23) and colon (21) carcinomas has prognostic value in patients with early-stage disease, indicating a relevant role for the mitochondrial H^+-ATP synthase in cancer progression and the potential utility of metabolic biomarkers in clinical oncology.

In this study we have analysed the bioenergetic signature of 101 breast carcinomas and 13 uninvolved breast samples by conventional immunological approaches. Quantitative measures of mitochondrial β-F1-ATPase, heat shock protein 60 (Hsp60), and of the glycolytic GAPDH, pyruvate kinase (PK) and lactate dehydrogenase (LDH), and of the ratios derived from them, were obtained and correlations in the expression level of the markers with the clinicopathological classification of the tumors and follow-up data of the patients were further established. The results obtained reveal a profound alteration in the expression of the selected markers of the mitochondrial and glycolytic proteomes strongly supporting that the alteration of the mitochondrial phenotype and concurrent phenotypic shift to glycolysis is also a hallmark feature of breast cancer. In addition, the results indicate that proteins which define the metabolic phenotype of the cell may help define the prognosis of breast cancer patients.

Materials and methods

Tissue specimens and clinical features of the patients

Samples from patients who had an operation for invasive breast carcinoma at the Hospital Universitario La Paz between 1991 and 2000 were snap-frozen in liquid nitrogen and stored at −70°C. The Institutional Review Board approved the project. Patients' medical records were reviewed, and identifiers coded to protect patient confidentiality. One hundred and one patients with stage I–III breast cancer, who had available full follow-up information and frozen tumor sample, and 13 uninvolved breast tissue samples, were retrospectively selected. Patients who received chemotherapy or radiotherapy before surgery were excluded. Table I summarizes patients' characteristics. Both mastectomy and lumpectomy were acceptable whenever negative surgical margins were achieved. Adjuvant radiotherapy was administered to all patients with a conservative procedure or those with four or more positive lymph nodes. Adjuvant chemotherapy was given in cases with positive lymph nodes or negative hormonal receptors or other poor-prognosis markers (such as grade 3 or size >5 cm). Sixty patients received cyclophosphamide, methotrexate, 5-fluorouracil (CMF) treatment adjuvant chemotherapy, 10 were treated with an anthracycline-containing regimen and 31 did not receive chemotherapy. Tamoxifen for 5 years was given to 76% of patients with positive hormonal receptors. Nineteen patients had distant metastases at first relapse, whereas twelve patients had loco-regional relapse. After a median follow-up of 69 months (range 8–127), the median overall survival (OS) has not been reached. Overall, the total number of patients that died or had recurrence of the disease during the follow-up period (127 months) was 17 and 31, respectively.

Preparation and protein extraction of the samples

Cryostat sections (5 μm) prepared from the samples to be used for protein isolation were stained with hematoxylin and analysed by an experienced breast pathologist. Eligible samples for the study had to include at least 90% of tumor

Table I. Clinicopathological characteristics and expression levels of metabolic markers in breast biopsies

Characteristics	No.	β-F1-ATPase	Hsp60	β-F1/Hsp60	GAPDH	PK	LDH	ΣGlycolytic	BEC–GAPDH
Normal	13	5.3 ± 0.6	0.11 ± 0.01	47.3 ± 4.9	0.18 ± 0.02	0.33 ± 0.08	0.20 ± 0.04	0.73 ± 0.13	285 ± 35
Tumour	101	4.7 ± 0.3	**0.85 ± 0.06**	**11.5 ± 1.8**	**0.76 ± 0.04**	**0.65 ± 0.03**	**0.55 ± 0.03**	**2.00 ± 0.10**	**26 ± 5**
Age									
<50	34	5.3 ± 0.6	0.88 ± 0.14	17.4 ± 4.8	0.75 ± 0.06	0.63 ± 0.06	0.55 ± 0.05	1.90 ± 0.12	37.1 ± 13.4
>50	67	4.4 ± 0.3	0.83 ± 0.06	8.5 ± 0.9	0.76 ± 0.05	0.67 ± 0.04	0.55 ± 0.04	2.00 ± 0.09	20.2 ± 4.6
Histology									
D	84	4.8 ± 0.3	0.88 ± 0.07	11.6 ± 2.1	0.78 ± 0.05	0.67 ± 0.04	0.58 ± 0.03	2.05 ± 0.08	24.9 ± 5.8
L	10	**3.6 ± 0.5**	**0.44 ± 0.13**	13.6 ± 3.1	0.64 ± 0.09	0.56 ± 0.09	**0.30 ± 0.03**	**1.45 ± 0.15**	45.7 ± 25.3
Others	7	5.5 ± 1.7	1.10 ± 0.30	7.3 ± 2.8	0.73 ± 0.09	0.61 ± 0.22	0.54 ± 0.12	2.00 ± 0.30	9.8 ± 2.6
No. nodes									
0	50	4.7 ± 0.4	0.78 ± 0.08	11.3 ± 1.9	0.69 ± 0.04	0.64 ± 0.05	0.52 ± 0.05	1.90 ± 0.10	24.0 ± 6.5
1–3	32	4.7 ± 0.4	0.82 ± 0.09	9.8 ± 1.9	0.87 ± 0.09	0.68 ± 0.05	0.57 ± 0.04	2.20 ± 0.10	16.8 ± 3.4
>3	19	4.6 ± 0.7	1.10 ± 0.20	15.1 ± 7.4	0.77 ± 0.09	0.63 ± 0.09	0.60 ± 0.06	2.00 ± 0.20	46.1 ± 22.6
Size									
≤20 mm	34	4.2 ± 0.4	0.74 ± 0.08	8.4 ± 1.1	0.66 ± 0.06	0.65 ± 0.06	0.65 ± 0.06	1.89 ± 0.12	19.5 ± 4.4
>20 mm	67	5.0 ± 0.4	0.90 ± 0.10	**13.1 ± 2.6**	0.81 ± 0.05	0.66 ± 0.04	0.55 ± 0.04	2.00 ± 0.10	**29.1 ± 7.9**
Stage									
I	21	3.7 ± 0.4	0.80 ± 0.10	7.9 ± 1.4	0.67 ± 0.07	0.58 ± 0.07	0.53 ± 0.07	1.80 ± 0.20	17.1 ± 4.7
II	57	**5.2 ± 0.4**	0.83 ± 0.07	11.8 ± 1.9	0.77 ± 0.05	0.70 ± 0.04	0.55 ± 0.04	2.05 ± 0.09	22.7 ± 5.7
III	23	4.3 ± 0.6	0.97 ± 0.19	14.1 ± 6.1	0.79 ± 0.09	0.60 ± 0.08	0.56 ± 0.05	1.90 ± 0.20	41.8 ± 18.8
Grade									
1	9	5.1 ± 1.4	0.50 ± 0.10	14.9 ± 4.9	0.71 ± 0.08	0.60 ± 0.06	0.46 ± 0.06	1.80 ± 0.10	24.1 ± 7.8
2	29	4.8 ± 0.5	0.60 ± 0.10	17.8 ± 5.2	0.74 ± 0.07	0.63 ± 0.06	0.52 ± 0.06	1.90 ± 0.10	36.1 ± 12.4
3	48	4.6 ± 0.3	**1.10 ± 0.10**	**7.0 ± 1.5**	0.83 ± 0.07	0.67 ± 0.05	0.60 ± 0.04	2.20 ± 0.10	17.2 ± 6.7
N/A	15	4.6 ± 0.8	0.63 ± 0.16	11.6 ± 2.2	0.50 ± 0.06	0.67 ± 0.10	0.50 ± 0.08	1.70 ± 0.20	35.1 ± 17.1
Hormonal receptor									
No	20	5.2 ± 0.7	0.89 ± 0.12	9.3 ± 2.8	0.66 ± 0.09	0.69 ± 0.09	0.61 ± 0.07	1.90 ± 0.20	18.5 ± 5.3
Yes	81	4.6 ± 0.3	0.83 ± 0.07	12.1 ± 2.1	0.79 ± 0.05	0.65 ± 0.04	0.55 ± 0.03	2.00 ± 0.10	27.7 ± 6.7
Chemotherapy									
No	31	4.7 ± 0.5	0.72 ± 0.09	11.9 ± 2.5	0.78 ± 0.07	0.67 ± 0.06	0.56 ± 0.13	2.00 ± 0.10	26.1 ± 9.9
Yes	70	4.7 ± 0.3	0.90 ± 0.10	11.4 ± 2.3	0.75 ± 0.05	0.65 ± 0.04	0.55 ± 0.03	1.98 ± 0.09	25.8 ± 6.6
Hormonotherapy									
No	40	4.8 ± 0.5	0.90 ± 0.10	14.8 ± 3.9	0.69 ± 0.05	0.58 ± 0.06	0.52 ± 0.05	1.80 ± 0.10	34.3 ± 10.9
Yes	61	4.6 ± 0.3	0.82 ± 0.07	9.4 ± 1.4	0.81 ± 0.06	0.71 ± 0.04	0.58 ± 0.04	**2.10 ± 0.10**	20.3 ± 5.5

No., Indicates the number of breast biopsies in each group. N/A, indicates not available. Tumor histological subtypes: D and L, for invasive ductal and lobular carcinomas, respectively. Others, mostly mixed invasive and lobular carcinoma. The expression levels of the markers are expressed in arbitrary units as mean ± SE. Bold typed text indicates a significance of $P < 0.050$ or less by Student's *t*-test when compared with normal, ductal, ≤20 mm, Stage I and grade 1 and 2, respectively.

cellularity. Following the criteria established by the pathologist, the regions of the tumors were specifically chosen that did not contain significant areas of fibrosis, inflammation, lymphocytes or necrosis, estimating that non-tumor cells contributed to <10% of the total cellular protein for the samples analysed. Breast and tumor tissue (~50–60 mg wet weight) were homogenized and solubilized in a buffer containing 7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate, 1% DTT, 1% ampholines (pI 3–10, Pharmacia/LKB), 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and a complete protease inhibitor cocktail (Roche Diagnostics GmbH) and processed as indicated previously (22). The insoluble material was removed by centrifugation (14 000 r.p.m.) at 4°C for 25 min. The protein concentration in the supernatants was determined with the Bradford reagent (Bio-Rad Protein Assay) using BSA as standard. Aliquots of the supernatants were stored at −80°C until used.

Determination of protein expression by western blotting

Normal and tumor samples (25 μg of protein) were fractionated on SDS–9% PAGE followed by immunoblot analysis (22) using the appropriate dilution of various antisera. A sample (25 μg of protein) of the Hs578T breast-cancer cell line was fractionated and processed in all the gels to allow the normalization of the signals obtained for the metabolic markers studied between the different gels that were performed. The antibodies used in this study included: rabbit anti-β-F_1-ATPase at a dilution 1:15 000 (21); mouse monoclonal anti-Hsp60 (SPA 807, Stressgene, Victoria, Canada) at a dilution 1:1000; goat polyclonal anti-muscle PK at a dilution 1:2000, mouse monoclonal anti-GAPDH at a dilution 1:20 000 and goat anti-lactate dehydrogenase at a dilution 1:1000, from Abcam (Cambridge, UK). Secondary horse-radish peroxidase conjugated goat anti-rabbit or anti-mouse or rabbit anti-goat antibody (1:5000 dilution) were used for detection, which was accomplished using an enhanced chemiluminescence detection method (Amersham Pharmacia Biotech, UK). Quantification of the intensity of the immunoreactive bands (arbitrary units) was accomplished using a Kodak DC120 Zoom digital camera and the Quantity One Software (Bio-Rad) package. To calculate the normalized expression level of β-F1-ATPase, the band intensity of β-F1-ATPase was divided by the band intensity of Hsp60 assayed for the same sample. To calculate the BEC indices (21) the aforementioned ratio was divided by the band intensity of GAPDH, PK, LDH or the sum of the three glycolytic proteins (ΣGlyc).

Immunohistochemistry

Sections of 5 μm were cut from formalin-fixed, paraffin-embedded tissue blocks. Blocks included invasive ductal carcinoma and adjacent non-tumoral ductal epithelium. Slides were deparaffined and endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in methanol for 10 min at room temperature. Antigens were retrieved by incubation in EDTA for 45 min at 155°C. The following primary antibodies were used: anti-β-F1-ATPase (1:3000), anti-GADPH (1:8000) and anti-Hsp60 (1:400). The antibodies were diluted in 1% BSA TBS. Tissue slides were incubated for 2 h at room temperature. Slides were then rinsed in TBS and incubated with the peroxidase-based EnVision™ kit (Dako Cytomation, Carpinteria, CA) for 30 min at room temperature. Specimens were then incubated with diaminobenzidine chromogenic substrate (Dako Cytomation) for 5 min at room temperature. Sections were counterstained with hematoxylin.

Statistical analysis

Distributions of molecular markers and other categorical variables were compared by χ^2 and Student's *t*-tests. The significance of linear regressions was assessed by Pearson's correlation *t*-test. Overall expression profiles of metabolic markers were analysed by unsupervised hierarchical clustering. For this purpose, data were reformatted by calculating the log(2) of the expression level of the marker in each sample relative to the mean expression level in normal samples. We used the Cluster Program from 'Expression Profiler Clustering home page' at http://ep.ebi.ac.uk/EP/EPCLUST using the Euclidean distances and complete linkage method. Hierarchical clustering with the squared Euclidean distances and centroid method was also carried out for the markers using the SPSS Software. The Fisher's linear discriminant function was used to assign the biopsy of the patients to one of two considered classes as previously indicated (23). The β-F1-ATPase/Hsp60 ratio and BEC–GAPDH index were used as discriminant variables. The actual error rate, or misclassification rate, was estimated by the Lachenbruch's 'holdout' procedure as previously detailed (23). To determine the association between the expression levels of the metabolic markers with OS and disease-free survival (DFS), the mean value of the parameters identified in the highly divergent cluster of breast tumors were used as cut points to define 'high' and 'low' risk groups. OS of patients was calculated from the date of tumor diagnosis. DFS was defined as the interval between the date of surgery and the date of tumor recurrence. Survival curves were derived from Kaplan–Meier estimates and compared by log-rank test. Cox proportional hazards regression methods were used to investigate the relationship between survival, clinical-pathological variables and protein expression in both univariate and multivariate models. Hazard ratios are presented with their 95% confidence intervals (95% CI). Statistical tests were two-sided at the 5% level of significance.

Results

Mitochondrial and glycolytic markers in breast cancer

Representative immunoblot analysis of the expression level of mitochondrial (β-F1-ATPase, Hsp60) and glycolytic (GAPDH, PK, LDH) marker proteins in samples of primary breast carcinomas and normal breast biopsies are illustrated in Figure 1A. The expression level of the markers in normal and tumor samples were normalized relative to the expression of the same marker found in the Hs578T breast cancer cell line, which was processed in all the gels for normalization of the signals obtained (Figure 1A). A sharp and significant increase in the expression level of the three glycolytic markers (GAPDH, PK and LDH) was observed in breast tumors when compared with normal breast (Figure 1A; Table I). Likewise, the expression of the mitochondrial Hsp60 also showed a significant increase in breast cancer when compared with normal (Figure 1A; Table I). We found no significant differences in the expression level of β-F1-ATPase in 101 tumor samples when compared with normal breast samples (Figure 1A; Table I). Consistent with these results the normalized expression level of β-F1-ATPase, as assessed by the β-F1/Hsp60 ratio, or by the BEC index (β-F1/Hsp60/GAPDH ratio), revealed a sharp and significant reduction in breast tumors when compared with normal (Table I).

Data on the expression level of the markers, the β-F1/Hsp60 ratio and the BEC–GAPDH index of the 101 breast carcinomas classified according to the different categories of the clinico-pathological variables available in this study are summarized in Table I. Compared with lobular carcinomas ductal carcinomas revealed a significantly higher expression level of β-F1-ATPase, Hsp60, LDH and the sum of glycolytic markers. Likewise, the expression of Hsp60 was significantly higher in poorly differentiated (Grade 3) tumors when compared with well (Grade 1) and moderately (Grade 2) differentiated ones (Table I). No other major differences between the proteomic variables and the clinical groups of tumors were observed.

An immunohistochemical analysis of the expression of β-F1-ATPase, Hsp60 and GAPDH markers of the BEC index was also carried out in several randomly selected breast cancer biopsies of the study. The specific granular staining of the cytoplasm of the normal ductal epithelial and carcinoma cells of the breast with anti-β-F_1-ATPase antibodies revealed no major changes in the expression of this mitochondrial marker (Figure 2A). However, it should be noted that in some of the biopsies analysed the β-F1-ATPase immunostaining was significantly diminished in those cells lying towards the inner core of the carcinoma when compared with the cells of the periphery of the carcinoma (Figure 2B). Hsp60 immunostaining of carcinoma cells was significantly augmented when compared with normal epithelial cells of the ducts of the same patient (Figure 2C). Unlike β-F1-ATPase immunostaining (Figure 2B), we observed no asymmetric immunostaining of Hsp60 within the carcinoma (data not shown). Likewise, the cytoplasmic GAPDH immunostaining of carcinoma cells was significantly augmented when compared with normal ductal cells in the same biopsy (Figure 2D). Overall, the

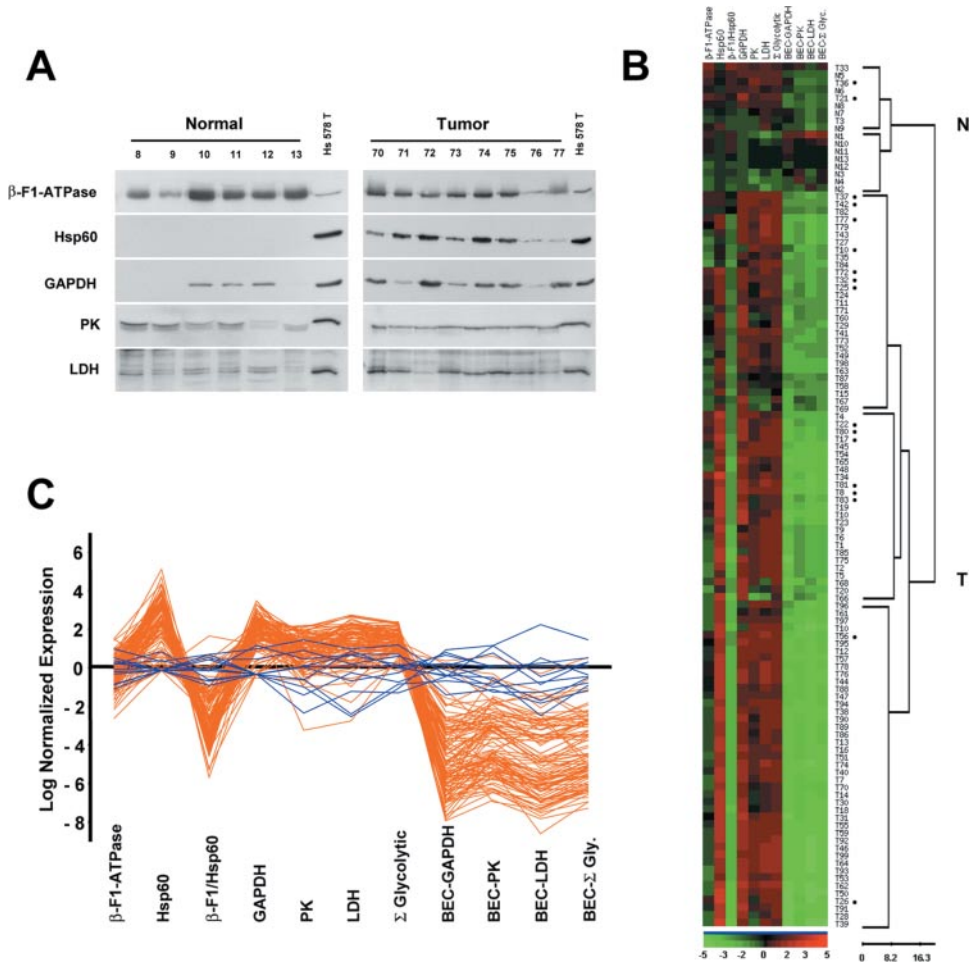

Fig. 1. Expression of mitochondrial and glycolytic markers in normal and breast cancer biopsies. (**A**) Representative western blot analysis of the expression levels of β-F1-ATPase, Hsp60, GAPDH, PK and LDH in SDS-PAGE fractionated proteins from normal (samples 8–13) and tumor (samples 70–77) breast biopsies. The expression level of the same markers in the Hs578T breast cancer cell line is also shown in the same gel. (**B**) Graphical unsupervised hierarchical clustering analysis of the overall mitochondrial and glycolytic phenotype in 114 breast biopsies (normal plus tumor) as measured by western blot. Rows, samples; columns, proteins and derived ratios. Protein expression scores are shown normalized to normal samples according to a color scale: red, high; black, normal and green, low. The dendogram (to the right of matrix) represents overall similarities in expression profiles providing four main different clusters, one for normal samples and three for tumors. The black dots, close to the sample identification number, identify the tumors with worse prognosis by the expression level of β-F1-ATPase (see Figure 4 for details). (**C**) The bioenergetic signature of breast cancer. Continuous color-coded lines connect the normalized expression level of the markers of the metabolic phenotype for each sample. Blue, normal; orange, tumors. The black line set at 0 level is the mean value of normal samples to which all the data have been normalized.

immunohistochemical results qualitatively confirmed those obtained by the large-scale immunoblotting procedure (Figure 1).

The bioenergetic signature of breast cancer

The overall proteomic expression pattern for the 114 biopsies (normal plus tumor samples) was analysed by unsupervised hierarchical clustering (Figure 1B and C). This analysis revealed two major groups of distribution for the biopsies, normal (N) and tumor (T), with none of the normal biopsies falling into the tumor group and only 3.5% of tumors falling in the normal group (Figure 1B). The tumor group was split into three clusters according to the degree of dissimilarity in the expression level of the markers when compared with normal biopsies (Figure 1B). Overall, these results (Figure 1B and C) indicate that carcinogenesis in the breast is almost invariably associated with an alteration of the mitochondrial proteome as revealed by the marked decrease in the β-F1/Hsp60 ratio (Figure 1B and C). It is likely that such alteration in the mitochondrial proteome would affect the overall energy transduction capability of the organelle. Consistent with a compromised oxidative phosphorylation in breast tumors, we observed a concurrent increase in the expression of the three cellular glycolytic markers (Figure 1B and C), therefore promoting a decrease in the BEC indices derived from each of them (Figure 1B and C). These results suggest the existence of a coordinate inverse relationship between mitochondrial bioenergetic function and glycolysis in breast carcinomas in agreement with similar findings in colorectal cancer (21), during development of the mammalian liver (27) and the differentiation of human myoblasts (28). In fact, significant inverse correlations were observed between the bioenergetic competence of the organelle (β-F1/Hsp60 ratio) and the glycolytic potential of the cell as assessed by the expression of GAPDH ($R = -0.370$, $P < 0.001$; data not shown), LDH ($R = -0.286$; $P < 0.003$; data not shown) and the sum of the expression level of the three glycolytic markers determined in the tumor samples ($R = -0.371$, $P < 0.001$; data not shown). Likewise, significant linear correlations were observed between the expression level of GAPDH and LDH

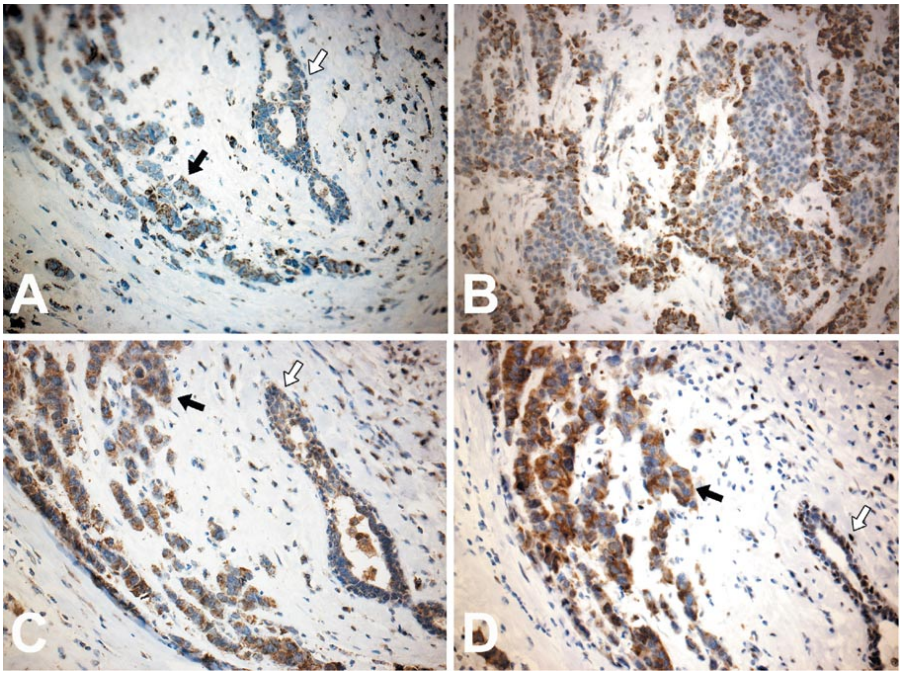

Fig. 2. Immunohistochemical analysis of β-F1-ATPase (**A** and **B**), Hsp60 (**C**) and GAPDH (**D**) expression in breast cancer. Representative photomicrographs provide examples of the immunostaining for each marker at ×20 (A, C and D) and ×40 (B) in the carcinoma (closed arrow) and in the proximal normal ductal epithelial cells (open arrow) of the same breast biopsy.

($R = 0.242$, $P < 0.014$; data not shown), PK and LDH ($R = 0.639$; $P < 0.001$; data not shown) and any of the glycolytic proteins (GAPDH, PK, LDH) with the sum of the expression level of the three glycolytic markers assayed in tumor biopsies ($R = 0.679$, $R = 0.741$ and $R = 0.797$, respectively; in all cases $P < 0.001$, not shown), indicating the concerted adaptation of the cancer cell to a glycolytic phenotype.

The Fisher linear discriminant analysis was applied using as predictor variables of the analysis the bioenergetic competence of mitochondria (β-F1/Hsp60 ratio) and its overall cellular competence (BEC–GAPDH index). Using cross-validation it was observed that the overall correct classification of the 114 biopsies studied was 95.6%, with a specificity of 85% and a sensitivity of 97%. In other words, the alteration of the mitochondrial proteome and concurrent induction of glycolytic markers is a definitive metabolic feature of the cancer cell phenotype that affects 97% of the breast tumors analysed.

Proteomic classification of breast carcinomas by the bioenergetic signature

As illustrated previously (Figure 1B), the combined protein expression patterns of the biopsies defined three major tumor clusters as revealed by the deviation of their bioenergetic signature from normal samples. As expected, hierarchical clustering of the 101 tumor samples by their overall expression patterns provided essentially the same findings; the tumors being classified into three clusters that were designated Low (L, $n = 30$), Medium (M, $n = 29$) and Highly (H, $n = 42$) divergent when compared with normal samples by their bioenergetic signature (Figure 3A). The three clusters revealed significant differences in the mean expression level of mitochondrial and glycolytic markers (see Figure 3B for a summary of some of them) suggesting a progressive alteration of the mitochondrial proteome and concurrent increase in the glycolytic potential of the tumors as divergence from normal augmented. The only relevant correlation between tumor clusters and biopathologic data observed was that tumors in cluster H grouped ∼2–4-fold higher number of the patients with poorly differentiated (Grade 3) tumors ($P = 0.009$, χ^2 test) when compared with tumors in clusters M and L, respectively. Kaplan–Meier survival analysis revealed that several of the variables used to define the bioenergetic signature of the tumors are significant predictors of both OS and DFS (Figure 3C). These observations independently suggest that the potential for cancer metastasis and recurrence in breast cancer is linked to the alteration of the mitochondrial proteome and the induction of the glycolytic potential of cancer cells.

The expression level of β-F1-ATPase in breast tumors discriminates patients with worse prognosis

The apparent lack of association between the expression of β-F1-ATPase in breast tumors and patients' survival contrasted with our previous findings in colon (21) and lung (23) cancer patients. We therefore proceeded to analyse the 101 tumor samples by unsupervised hierarchical clustering based on the expression level of β-F1-ATPase alone. We found that the expression level of β-F1-ATPase allowed the discrimination of two main groups of breast tumors: cluster C1, which contained 81% of the tumors, and cluster C2, which contained 17% of them (Figure 4A). Surprisingly, tumors in cluster C2 had a much higher expression level of β-F1-ATPase than normal breast biopsies and cluster C1 tumors (Figure 4A). Other independent proteomic variables were not significantly different between C1 and C2 tumors (Table II) being essentially the same as those reported for all tumors (Table I). Remarkably, it was found that the majority (∼90%) of the tumors in cluster C2 corresponded to tumors classified in the Low and Medium divergent clusters by their overall bioenergetic signature (see Figure 1B for details, and Figure 3A). Review of the clinicopathological differences in C1 and C2

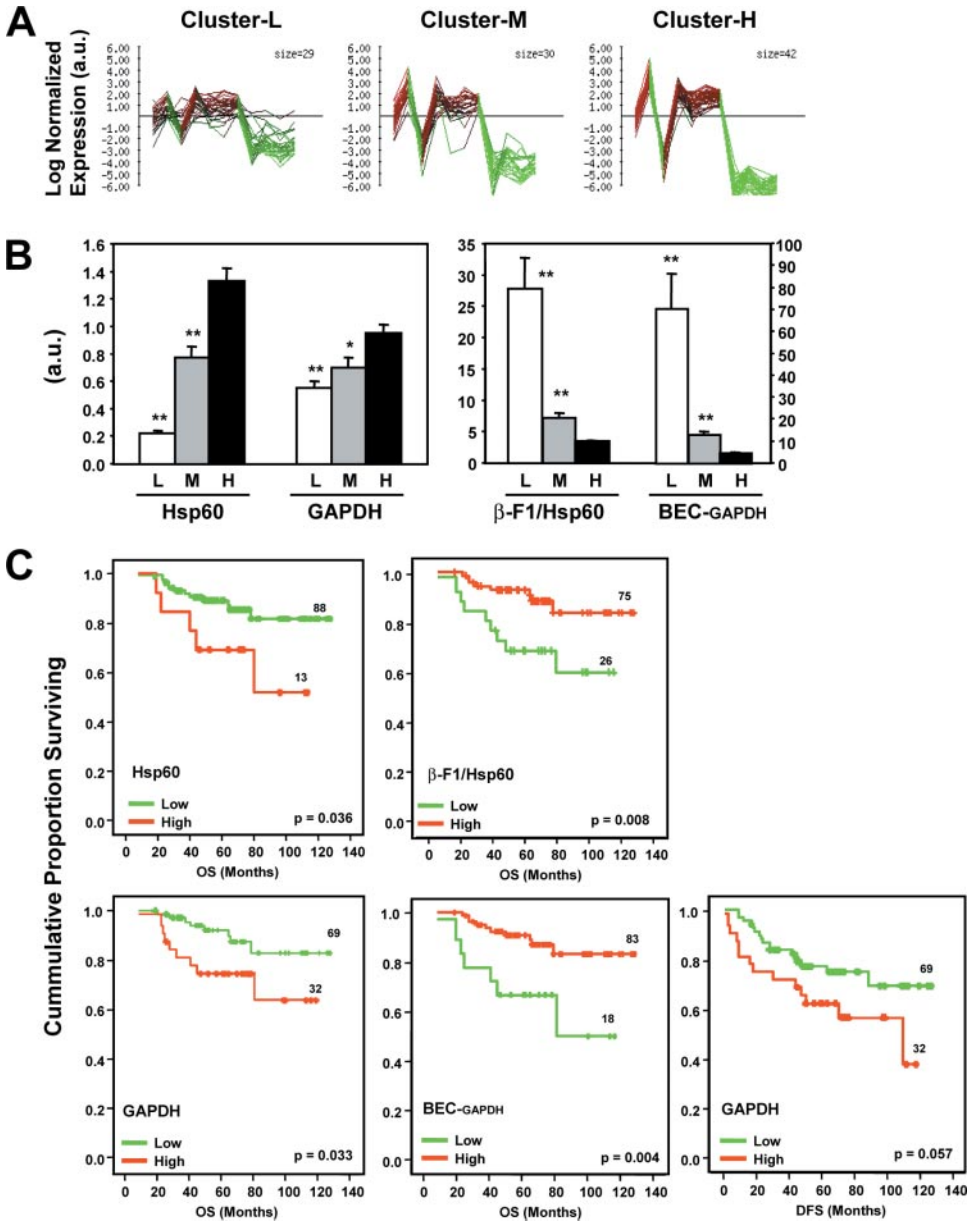

Fig. 3. Classification of 101 breast cancers by the bioenergetic signature and survival analysis by markers of the metabolic phenotype. (**A**) Unsupervised hierarchical clustering analysis of the overall mitochondrial and glycolytic phenotypes in 101 breast cancer biopsies provided three tumor clusters that were designated Low (L, $n = 29$), Medium (M, $n = 30$) and Highly (H, $n = 42$) divergent when compared with normal samples by their bioenergetic signature (for other details see Figure 1). (**B**) The histograms summarize the mean expression level of each marker (bars, SE) as well as the calculated β-F1-ATPase/Hsp60 and BEC–GAPDH ratios in cluster-L (open bars), cluster-M (gray bars) and cluster-H (closed bars) tumors. $^{*}P < 0.05$ and $^{**}P < 0.001$ when compared with cluster-H by Student's *t*-test. (**C**) The Kaplan–Meier survival analysis shows the association of the expression level of the indicated markers in breast carcinomas with overall survival (OS) and disease-free survival (DFS). Patients (numbers on top of each trace) were stratified in high (red) and low (green) expression levels by the mean value of the corresponding marker in cluster-H tumors. Log-rank significance is indicated in each plot.

clusters revealed that patients' age, clinical stage, histological type and grade, nodal and hormonal receptor status, tumor size and adjuvant therapies were not different (Table II). In contrast, >2-fold differences were observed in the percentage of the patients that had recurrence of the disease between the C1 and C2 clusters (Table II). Therefore, it was found that patients that belong to the C1 cluster had a significant advantage in both OS (Figure 4B) and DFS (Figure 4C) when compared with patients in cluster C2 by Kaplan–Meier analysis. In other words, of the 48 patients in this study who had >5-year metastasis-free survival only 8% corresponded to the poor-prognosis group whereas 92% of them belonged to the good-prognosis group. Univariate Cox regression analysis further confirmed the significant association of the cluster analysis by the expression level of β-F1-ATPase with OS and DFS among the patients with breast cancer revealing a 3-fold higher relative risk for the patients in the C2 cluster (Table III). A similar approach with the other proteomic variables of the study provided no significant findings.

Association of clinical and proteomic variables with disease-free and overall patient survival

Two clinical-pathological variables, nodal affectation and tumor size, had a significant and marginal relationship with OS (Table III). Of the proteomic variables, the expression of Hsp60 and β-F1-ATPase clustering significantly correlated

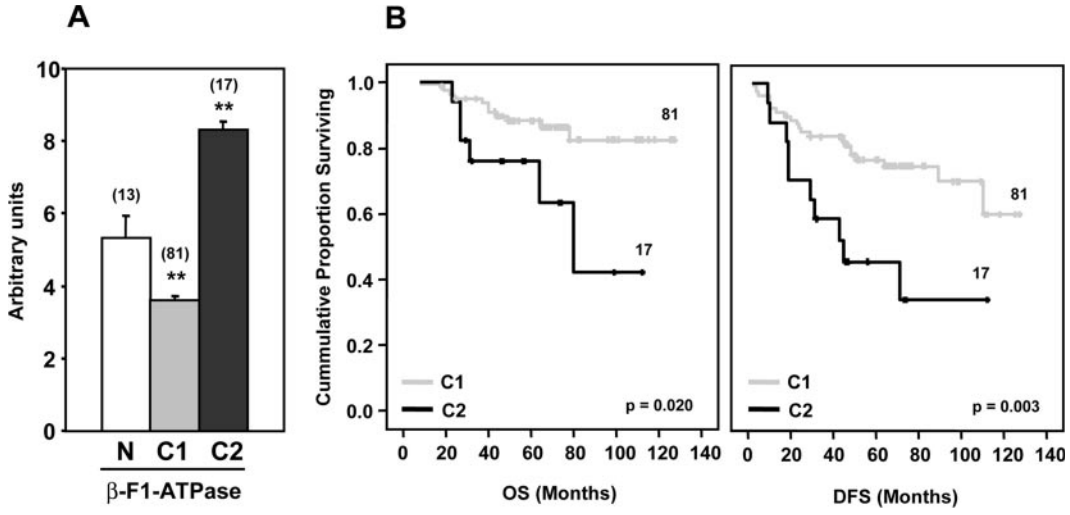

Fig. 4. Classification of breast carcinomas by the expression of β-F1-ATPase and survival analysis. Hierarchical clustering analysis of 101 breast cancer biopsies by the expression level of β-F1-ATPase provided two major tumor clusters that were designated C1($n = 81$) and C2 ($n = 17$). (**A**) The histograms summarize the mean expression level of β-F1-ATPase (bars, SE) in normal biopsies (N) and in clusters C1 and C2. $^{**}P < 0.001$ when compared with N by Student's *t*-test. (**B**) The Kaplan–Meier survival analysis shows the association of the β-F1 clustering with OS and DFS in breast cancer patients. Log-rank significance is indicated in each plot.

with OS, whereas GAPDH expression had a marginal significance (Table III). On multivariate Cox regression models it was found that only β-F1-ATPase clustering provided additional significant and independent information to the degree of nodal affectation and tumor size in the prognosis of the patients OS (Table III). Likewise, univariate Cox regression analysis revealed that two clinical-pathological variables, nodal affectation and clinical stage, significantly correlated with DFS (Table III). Of the proteomic variables, while the expression of GAPDH marginally correlated with DFS it was found that β-F1-ATPase clustering significantly correlated with DFS (Table III). On multivariate Cox regression models it was found that β-F1-ATPase clustering provided additional independent information to the clinical stage for the prognosis of disease recurrence in the patients (Table III). Patient's age, histological type and tumor grade, tumor hormonal receptor status, adjuvant chemotherapy, radiotherapy and hormonotherapy, β-F1/Hsp60, PK, LDH and BEC indices were not significantly correlated with survival by Cox regression analysis. We should note that the survival analysis performed at 70 and 100 months of the follow-up period (data not shown) provided essentially the same findings as those summarized under Figures 3 and 4, and Table III.

Discussion

Until recently, the 'aerobic glycolytic' metabolism of cancer cells (29) had received little attention in molecular oncology despite being one of the first observations made in the cancer field (30), because it was considered an epiphenomenon of cell transformation (6). Nowadays, mounting functional and molecular evidence indicates that such interpretation cannot be sustained any longer because of the molecular and functional relationships that exist between energetic metabolism and apoptosis (31–35). Our results in breast cancer illustrate that the majority of carcinomas revealed a profound shift towards a glycolytic phenotype by the concerted increased expression of three molecular markers of the glycolytic pathway. The molecular mechanisms that promote the expression of an aberrant aerobic metabolic phenotype in tumors and cancer cells are nowadays a subject of renewed debate. In this regard, it is a widely accepted view that induction of the glycolytic phenotype in the tumor could represent a reactive response to the hypoxic environment where the tumor develops (36) and indeed, a significant fraction of breast tumors do reveal overexpression of HIF1α (37). In this regard, it is tempting to suggest that the decreased expression of β-F1-ATPase observed in the inner core of some carcinomas (Figure 2B) might result from a partial hypoxic environment in such tumors since the expression of this protein is stringently controlled by the cellular redox and adenylate energy state (38) at the level of mRNA translation (20,39). On the other hand, other authors support the view that the phenotypic shift to glycolysis results from oncogenic mutations on elements of the signal transduction pathways that control cellular glucose uptake (40) or on transcription factors that control the expression of glycolytic genes (41). Moreover, and in line with the original formulation of the Warburg hypothesis, several authors have described the linkage of mutations on mitochondrial and nuclear genes that participate on energy transduction in a large variety of carcinomas (11–13). Interestingly, some of the described mutations on mtDNA have been recently confirmed to contribute to the promotion of cancer (17,18,35). Moreover, mutations on nuclear genes that impact on the activity of succinate dehydrogenase (14–16), a component of the Krebs cycle and of the respiratory chain, and therefore participate in mitochondrial energy transduction have been recently associated with the accumulation of a metabolic intermediate (succinate) that inhibits HIF-1α prolyl-hydroxylase (42), contributing in this way to the abnormal glycolytic phenotype of cancer cells. The results in this work do support that the glycolytic shift of breast cancers also results from an impaired mitochondrial function because, as revealed by Fisher's and cluster analysis, it is invariably associated with a profound alteration of the mitochondrial proteome of the breast cancer cell in agreement with previous findings in other human cancers (21–26). The alteration of the mitochondrial proteome in cancer strongly suggests that progression of human neoplasias implicate alterations on the mechanisms that

Table II. Clinicopathological characteristics and expression level of metabolic markers in C1 and C2 β-F1-ATPase breast tumor clusters

β-F1-ATPase cluster					χ^2	*P*-value
Characteristics	C1		C2			
	n	Value	*n*	Value		
Categorical						
Grade					0.328	0.849
1	7	10.3	1	6.3		
2	22	32.4	6	37.5		
3	39	57.4	9	56.3		
No. nodes					0.961	0.618
0	40	49.4	8	47.1		
1–3	25	30.9	7	41.2		
>3	16	19.8	2	11.8		
Age					1.941	0.164
<50	24	29.6	8	47.1		
>50	57	70.4	9	52.9		
Size					0.253	0.615
<20 mm	29	35.8	5	29.4		
>20 mm	52	64.2	12	70.6		
Stage					3.460	0.177
I	19	23.5	2	11.8		
II	42	51.9	13	76.5		
III	20	24.7	2	11.8		
Histology					2.362	0.124
DI	66	86.8	16	100		
LI	10	13.2	0	0		
Hormonal receptor					0.226	0.635
Negative	15	18.5	4	23.5		
Positive	66	81.5	13	76.5		
Relapse					7.032	**0.008**
No	60	74.1	7	41.2		
Yes	21	25.9	10	58.8		
Numerical						
β-F1-ATPase	81	3.6 ± 0.1	17	8.3 ± 0.2		**0.001**
Hsp60	81	0.8 ± 0.1	17	0.9 ± 0.2		0.955
GAPDH	81	0.7 ± 0.0	17	0.9 ± 0.1		0.201
PK	73	0.6 ± 0.0	17	0.7 ± 0.1		0.424
LDH	73	0.5 ± 0.0	17	0.6 ± 0.1		0.367
Σ Glycolytic	73	2.0 ± 0.1	17	2.2 ± 0.1		0.096
β-F1/Hsp60	81	7.8 ± 0.9	17	21.6 ± 4.9		**0.012**
BEC–GAPDH	81	18.7 ± 3.9	17	42.1 ± 17.8		0.216

Values in the categorical variables are expressed as percentage of the tumors in that cluster. Categorical variables were compared by χ^2. The expression level of the metabolic markers are expressed in arbitrary units as mean ± SE. Bold type text indicates a significance of $P < 0.050$ when compared with cluster C1.

regulate the cell-type specific programs that control the differentiation and/or proliferation of mitochondria in that particular tissue (21,22,27). In any case, and irrespective of the underlying molecular mechanism(s) that could promote the abrupt change in the bioenergetic signature of breast cancer, what is clear from this study and previous findings in other carcinomas (21,23) is that the metabolic phenotype is providing a set of molecular markers with potential utility in the prognosis and future development of new treatment strategies of cancer patients.

Breast cancer is a heterogeneous disease in which molecular alterations could impact on any of the hallmark features of the cancer cell (43). Despite this heterogeneity, the phenotypic presentation of breast cancer is invariably associated with an alteration of the bioenergetic signature of the tumor cell allowing the discrimination of normal and tumor biopsies with sensitivity >97%. This figure is higher than that obtained for most 'promising' prognostic biological factors in breast cancer, such as those implicated in self-sufficiency in growth signals (c-myc, HER/erbB), insensitivity to anti-growth signals (cyclins, p53), evasion of apoptosis (bcl-2), sustained angiogenesis (VEGF, HIF-1α, CD31/PECAM-1) and tissue invasion, and metastasis (uPA/PAI-1) (for review see ref. (5)). This finding strongly suggests the potential utility of some of the biomarkers involved in the bioenergetic signature as promising general prognostic indicators of breast cancer and perhaps, as potential predictive markers of therapeutic intervention. In this regard, it appears that some of the markers of the metabolic phenotype such as Hsp60, GAPDH, β-F1/Hsp60 ratio and BEC–GAPDH index, provided significant markers of OS and DFS of the patients (Figure 3). However, we suggest that the expression level of these markers reveals, at the proteome level, disease-related patient's characteristics. In fact, an increased expression of Hsp60 is noted in tumors of advanced clinical stage when compared with earlier stage carcinomas (Table I). This finding is further supported and confirmed by the analysis of the overall expression of the metabolic markers (Figure 3) which identified tumors in advanced stages of the disease by their large divergence from the normal metabolic signature. Furthermore, in multivariate analysis these markers failed to provide additional information to the variables included in the TNM system (Table III). However, they do offer a quantitative estimate to assess the extension of the disease. In any case, these results do suggest that progressive alteration of the mitochondrial proteome and concurrent increase in the glycolytic potential of breast tumors is also a required metabolic condition for cancer progression.

In contrast to the above findings that indicated a progressive alteration of the mitochondrial/glycolytic proteome in breast cancer with disease progression, we observed that the expression level of β-F1-ATPase alone identified a subgroup of breast cancer patients with significant worse prognosis both in terms of OS and recurrence of the disease (Figure 4). The surprising finding was that these patients had a higher expression level of the mitochondrial marker of energy transduction than that observed in normal samples. Multivariate models suggested that clustering by the expression level of β-F1-ATPase is a significant prognostic marker independent from clinical variables. In agreement with this observation, most of the breast tumors identified (~90%) by this marker in the worse prognosis group fall within the group of tumors that revealed less divergence in their overall bioenergetic signature from normal samples (Figure 1). These results strongly support the potential utility of β-F1-ATPase expression as an independent proteomic marker for the identification of breast cancer patients with worse prognosis, especially among the group of early-stage patients that have a lesser alteration of the bioenergetic signature of the tumor. However, they raise the intriguing possibility of the potential dual implication of mitochondrial bioenergetics in breast cancer progression.

It appears that mitochondrial oxidative phosphorylation is required for efficient execution of apoptosis. In fact, cells devoid of mtDNA (ρ°), which are unable to carry on oxidative phosphorylation, have a resistant apoptotic phenotype (35,44–46). Likewise, oligomycin, a specific inhibitor of the mitochondrial H^+-ATP synthase, halts the efficient execution of apoptosis (47). In addition, the activity of oxidative phosphorylation has been shown to be required for Bax induced toxicity in yeast cells (48) and genetic screens in yeast, aimed at the identification of genes that could confer a Bax-resistance phenotype, allowed the identification of a subunit of the

Table III. Cox proportional hazards univariate and multivariate analysis in overall survival (OS) and disease-free survival (DFS)

Model	Variables	HR (95% CI)	*P*
OS			
Univariate	Continuous		
	Hsp60	1.7 (1.0–2.9)	**0.041**
	GAPDH	2.8 (0.9–8.9)	0.072
	Categorical		
	Nodes		
	0	1	
	0 versus 1–3	3.5 (0.88–14.1)	0.076
	0 versus >3	8.5 (2.25–32)	**0.002**
	Size		
	<20 mm	1	
	<20 mm versus >20 mm	4.0 (0.9–17.4)	0.067
	Stage		
	I	1	
	I versus II	3200 (0–. . .)	0.915
	I versus III	97000 (0–. . .)	0.906
	Cluster βF1		
	C1	1	
	C1 versus C2	3.2 (1.2–8.7)	**0.023**
Multivariate	Nodes/Cluster βF1		
	Nodes		
	0	1	
	0 versus 1–3	3.1 (0.8–12.4)	0.111
	0 versus >3	9.0 (2.4–34.0)	**0.001**
	Cluster βF1		
	C1	1	
	C1 versus C2	3.2 (1.2–8.8)	**0.023**
	Size/Cluster βF1		
	Size		
	<20 mm	1	
	<20 mm versus >20 mm	4.0 (0.9–17.4)	0.068
	Cluster βF1		
	C1	1	
	C1 versus C2	3.0 (1.1–8.1)	**0.033**
DFS			
Univariate	Continuous		
	Hsp60	1.2 (0.7–1.9)	0.489
	GAPDH	2.2 (0.9–5.2)	0.068
	Categorical		
	Nodes		
	0	1	
	0 versus 1–3	2.8 (1.1–6.8)	**0.026**
	0 versus >3	5.2 (2.1–12.9)	**<0.001**
	Size		
	<20 mm	1	
	<20 mm versus >20 mm	1.6 (0.7–3.7)	0.228
	Stage		
	I	1	
	I versus II	3.5 (0.8–15.3)	0.094
	I versus III	9.1 (2.0–40.3)	**0.004**
	Cluster βF1		
	C1	1	
	C1 versus C2	3.0 (1.4–6.4)	**0.005**
Multivariate	Stage/GAPDH		
	Stage		
	I	1	
	I versus II	3.2 (0.7–13.8)	0.127
	I versus III	8.8 (2.0–39.3)	**0.004**
	GAPDH	2.2 (0.9–5.1)	0.066
	Stage/Cluster βF1		
	Stage		
	I	1	
	I versus II	3.3 (0.7–14.4)	0.114
	I versus III	10.1 (2.3–45.1)	**0.002**
	Cluster βF1		
	C1	1	
	C1 versus C2	3.2 (1.5–6.8)	**0.003**

Bold typed text indicates a significance of $P < 0.05$ by Wald test.

mitochondrial H^+-ATP synthase critical for Bax-mediating killing of *Saccharomyces cerevisiae* (47). Moreover, Bax-mediated killing of the budding yeast has been shown to be strictly dependent upon select mitochondrial components such as the nuclear encoded β-F1-ATPase (49). Therefore, it seems reasonable to suggest that the progressive alteration of the bioenergetic signature observed in breast carcinomas (this study) as well as in other human cancers (21–23) and in rat hepatocarcinomas (20) might contribute to tumor progression by diminishing the apoptotic potential of the cancer cell. Based on these grounds, the question now is why a higher-than-normal expression level of β-F1-ATPase predicts an unfavorable patient outcome in breast cancers. A possibility could arise from differences in the bioenergetic phenotype of normal breast cells as dictated by differences in the human genetic, metabolic and/or environmental background. In fact, it is well established that the incidence of breast cancer is different depending upon ethnic groups and/or nutritional and social habits (1). An alternative possibility is that the genetic alterations acquired in this group of patients directly impact on components of cellular energy transduction, blurring in this way the beneficial contribution that mitochondrial oxidative phosphorylation could provide to the overall apoptotic potential of the cell. In any case, we suggest that further retrospective and prospective studies are required to explore the molecular causes that defined the worse prognostic group of breast cancer patients by the expression level of β-F1-ATPase. Finally, recent promising findings in animal models (50,51) and cells in culture (35) indicate that targeting the energetic metabolism of the tumors might provide a new approach to cancer treatment. In this regard, we would like to stress that the bioenergetic signature of the tumor may provide a convenient tool in the clinical setting to establish future chemotherapeutic strategies aimed at targeting the energy provision pathway in breast cancer patients.

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References

1. Stewart,B.W. and Kleihues,P. (2003) *World Cancer Report*. IARC Press, pp. 188–193.
2. Eeles,R.A. (1999) Screening for hereditary cancer and genetic testing, epitomized by breast cancer. *Eur. J. Cancer*, **35**, 1954–1962.
3. Goldhirsch,A., Wood,W.C., Gelber,R.D., Coates,A.S., Thurlimann,B. and Senn,H.J. (2003) Meeting highlights: updated international expert consensus on the primary therapy of early breast cancer. *J. Clin. Oncol.*, **21**, 3357–3365.
4. Singletary,S.E., Allred,C., Ashley,P. *et al.* (2003) Staging system for breast cancer: revisions for the 6th edition of the AJCC Cancer Staging Manual. *Surg. Clin. North Am.*, **83**, 803–819.
5. Coradini,D. and Daidone,M.G. (2004) Biomolecular prognostic factors in breast cancer. *Curr. Opin. Obstet. Gynecol.*, **16**, 49–55.
6. Garber,K. (2004) Energy boost: the Warburg effect returns in a new theory of cancer. *J. Natl Cancer Inst.*, **96**, 1805–1806.
7. Perry,M.E., Dang,C.V., Hockenbery,D. and Moll,U. (2004) Highlights of the National Cancer Institute Workshop on mitochondrial function and cancer. *Cancer Res.*, **64**, 7640–7644.

8. van de Vijver,M.J., He,Y.D., van't Veer,L.J. *et al*. (2002) A gene-expression signature as a predictor of survival in breast cancer. *N. Engl. J. Med*., **347**, 1999–2009.
9. Verma,M., Kagan,J., Sidransky,D. and Srivastava,S. (2003) Proteomic analysis of cancer-cell mitochondria. *Nat. Rev. Cancer*, **3**, 789–795.
10. Jacquemier,J., Ginestier,C., Rougemont,J. *et al*. (2005) Protein expression profiling identifies subclasses of breast cancer and predicts prognosis. *Cancer Res*., **65**, 767–779.
11. Parrella,P., Xiao,Y., Fliss,M. *et al*. (2001) Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates. *Cancer Res*., **61**, 7623–7626.
12. Tan,D.J., Bai,R.K. and Wong,L.J. (2002) Comprehensive scanning of somatic mitochondrial DNA mutations in breast cancer. *Cancer Res*., **62**, 972–976.
13. Carew,J.S. and Huang,P. (2002) Mitochondrial defects in cancer. *Mol. Cancer*, **1**, 1–9.
14. Baysal,B.E., Ferrell,R.E., Willett-Brozick,J.E. *et al*. (2000) Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. *Science*, **287**, 848–851.
15. Habano,W., Sugai,T., Nakamura,S., Uesugi,N., Higuchi,T., Terashima,M. and Horiuchi,S. (2003) Reduced expression and loss of heterozygosity of the SDHD gene in colorectal and gastric cancer. *Oncol. Rep*., **10**, 1375–1380.
16. Neumann,H.P., Pawlu,C., Peczkowska,M. *et al*. (2004) Distinct clinical features of paraganglioma syndromes associated with SDHB and SDHD gene mutations. *JAMA*, **292**, 943–951.
17. Petros,J.A., Baumann,A.K., Ruiz-Pesini,E. *et al*. (2005) mtDNA mutations increase tumorigenicity in prostate cancer. *Proc. Natl Acad. Sci. USA*, **102**, 719–724.
18. Shidara,Y., Yamagata,K., Kanamori,T., Nakano,K., Kwong,J.Q., Manfredi,G., Oda,H. and Ohta,S. (2005) Positive contribution of pathogenic mutations in the mitochondrial genome to the promotion of cancer by prevention from apoptosis. *Cancer Res*., **65**, 1655–1663.
19. Amuthan,G., Biswas,G., Zhang,S.Y., Klein-Szanto,A., Vijayasarathy,C. and Avadhani,N.G. (2001) Mitochondria-to-nucleus stress signaling induces phenotypic changes, tumor progression and cell invasion. *EMBO J*., **20**, 1910–1920.
20. López de Heredia,M., Izquierdo,J.M., and Cuezva,J.M. (2000) A conserved mechanism for controlling the translation of β-F1-ATPase mRNA between the fetal liver and cancer cells. *J. Biol. Chem*., **275**, 7430–7437.
21. Cuezva,J.M., Krajewska,M., López de Heredia,M., Krajewski,S., Santamaría,G., Kim,H., Zapata,J.M., Marusawa, Chamorro,M. and Reed,J.C. (2002) The bioenergetic signature of cancer: a marker of tumor progression. *Cancer Res*., **62**, 6674–6681.
22. Isidoro,A., Martinez,M., Fernandez,P.L., Ortega,A.D., Santamaria,G., Chamorro,M., Reed,J.C. and Cuezva,J.M. (2004) Alteration of the bioenergetic phenotype of mitochondria is a hallmark of breast, gastric, lung and oesophageal cancer. *Biochem. J*., **378**, 17–20.
23. Cuezva,J.M., Chen,G., Alonso,A.M., Isidoro,A., Misek,D.E., Hanash,S.M. and Beer,D.G. (2004) The bioenergetic signature of lung adenocarcinomas is a molecular marker of cancer diagnosis and prognosis. *Carcinogenesis*, **25**, 1157–1163.
24. Unwin,R.D., Craven,R.A., Harnden,P., Hanrahan,S., Totty,N., Knowles,M., Eardley,I., Selby,P.J. and Banks,R.E. (2003) Proteomic changes in renal cancer and co-ordinate demonstration of both the glycolytic and mitochondrial aspects of the Warburg effect. *Proteomics*, **3**, 1620–1632.
25. Yin,P.H., Lee,H.C., Chau,G.Y., Wu,Y.T., Li,S.H., Lui,W.Y., Wei,Y.H., Liu,T.Y. and Chi,C.W. (2004) Alteration of the copy number and deletion of mitochondrial DNA in human hepatocellular carcinoma. *Br. J. Cancer*, **90**, 2390–2396.
26. He,Q.Y., Chen,J., Kung,H.F., Yuen,A.P. and Chiu,J.F. (2004) Identification of tumor-associated proteins in oral tongue squamous cell carcinoma by proteomics. *Proteomics*, **4**, 271–278.
27. Cuezva,J.M., Ostronoff,L.K., Ricart,J., López de Heredia,M., Di Liegro,C.M. and Izquierdo,J.M. (1997) Mitochondrial biogenesis in the liver during development and oncogenesis. *J. Bioenerg. Biomembr*., **29**, 365–377.
28. Webster,K.A., Gunning,P., Hardeman,E., Wallace,D.C. and Kedes,L. (1990) Coordinate reciprocal trends in glycolytic and mitochondrial transcript accumulations during the in vitro differentiation of human myoblasts. *J. Cell. Physiol*., **142**, 566–573.
29. Pedersen,P.L. (1978) Tumor mitochondria and the bioenergetics of cancer cells. *Prog. Exp. Tumor Res*., **22**, 190–274.
30. Warburg,O. (1956) On the origin of cancer cells. *Science*, **123**, 309–314.
31. Plas,D.R. and Thompson,C.B. (2002) Cell metabolism in the regulation of programmed cell death. *Trends Endocrinol. Metab*., **13**, 75–78.
32. Pedersen,P.L., Mathupala,S., Rempel,A., Geschwind,J.F. and Ko,Y.H. (2002) Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention. *Biochim. Biophys. Acta*, **1555**, 14–20.
33. Danial,N.N., Gramm,C.F., Scorrano,L. *et al*. (2003) BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. *Nature*, **424**, 952–956.
34. Azoulay-Zohar,H., Israelson,A., Abu-Hamad,S. and Shoshan-Barmatz,V. (2004) In self-defence: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death. *Biochem. J*., **377**, 347–355.
35. Xu,R.H., Pelicano,H., Zhou,Y., Carew,J.S., Feng,L., Bhalla,K.N., Keating,M.J. and Huang,P. (2005) Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. *Cancer Res*., **65**, 613–621.
36. Semenza,G.L., Artemov,D., Bedi,A. *et al*. (2001) 'The metabolism of tumours': 70 years later. *Novartis Found. Symp*., **240**, 251–260.
37. Bos,R., Zhong,H., Hanrahan,C.F., Mommers,E.C., Semenza,G.L., Pinedo,H.M., Abeloff,M.D., Simons,J.W., van Diest,P.J. and van der Wall,E. (2001) Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. *J. Natl Cancer Inst*., **93**, 309–314.
38. Izquierdo,J.M. and Cuezva,J.M. (2005) Epigenetic regulation of the binding activity of translation inhibitory proteins that bind the 3′ untranslated region of beta-F1-ATPase mRNA by adenine nucleotides and the redox state. *Arch. Biochem. Biophys*., **433**, 481–486.
39. Izquierdo,J.M. and Cuezva,J.M. (1997) Control of the translational efficiency of beta-F1-ATPase mRNA depends on the regulation of a protein that binds the 3' untranslated region of the mRNA. *Mol. Cell. Biol*., **17**, 5255–5268.
40. Elstrom,R.L., Bauer,D.E., Buzzai,M. *et al*. (2004) Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res*., **64**, 3892–3899.
41. Osthus,R.C., Shim,H., Kim,S., Li,Q., Reddy,R., Mukherjee,M., Xu,Y., Wonsey,D., Lee,L.A. and Dang,C.V. (2000) Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *J. Biol. Chem*., **275**, 21797–21800.
42. Selak,M.A., Armour,S.M., MacKenzie,E.D., Boulahbel,H., Watson,D.G., Mansfield,K.D., Pan,Y., Simon,M.C., Thompson,C.B. and Gottlieb,E. (2005) Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. *Cancer Cell*, **7**, 77–85.
43. Hanahan,D. and Weinberg,R.A. (2000) The hallmarks of cancer. *Cell*, **100**, 57–70.
44. Dey,R. and Moraes,C.T. (2000) Lack of oxidative phosphorylation and low mitochondrial membrane potential decrease susceptibility to apoptosis and do not modulate the protective effect of Bcl-x(L) in osteosarcoma cells. *J. Biol. Chem*., **275**, 7087–7094.
45. Kim,J.Y., Kim,Y.H., Chang,I., Kim,S., Pak,Y.K., Oh,B.H., Yagita,H., Jung,Y.K., Oh,Y.J. and Lee,M.S. (2002) Resistance of mitochondrial DNA-deficient cells to TRAIL: role of Bax in TRAIL-induced apoptosis. *Oncogene*, **21**, 3139–3148.
46. Park,S.Y., Chang,I., Kim,J.Y., Kang,S.W., Park,S.H., Singh,K. and Lee,M.S. (2004) Resistance of mitochondrial DNA-depleted cells against cell death: role of mitochondrial superoxide dismutase. *J. Biol. Chem*., **279**, 7512–7520.
47. Matsuyama,S., Xu,Q., Velours,J. and Reed,J.C. (1998) The mitochondrial F0F1-ATPase proton pump is required for function of the proapoptotic protein Bax in yeast and mammalian cells. *Mol. Cell*, **1**, 327–336.
48. Harris,M.H., Vander Heiden,M.G., Kron,S.J. and Thompson,C.B. (2000) Role of oxidative phosphorylation in Bax toxicity. *Mol. Cell. Biol*., **20**, 3590–3596.
49. Gross,A., Pilcher,K., Blachly-Dyson,E., Basso,E., Jockel,J., Bassik,M.C., Korsmeyer,S.J. and Forte,M. (2000) Biochemical and genetic analysis of the mitochondrial response of yeast to BAX and BCL-X(L). *Mol. Cell. Biol*., **20**, 3125–3136.
50. Geschwind,J.F., Ko,Y.H., Torbenson,M.S., Magee,C. and Pedersen,P.L. (2002) Novel therapy for liver cancer: direct intraarterial injection of a potent inhibitor of ATP production. *Cancer Res*., **62**, 3909–3913.
51. Ko,Y.H., Smith,B.L., Wang,Y., Pomper,M.G., Rini,D.A., Torbenson,M.S., Hullihen,J. and Pedersen,P.L. (2004) Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP. *Biochem. Biophys. Res. Commun*., **324**, 269–275.

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