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The bioenergetic signature of lung adenocarcinomas is a molecular marker of cancer diagnosis and prognosis

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The aim of this study was to investigate the mitochondrial bioenergetic signature of lung adenocarcinomas as a prognostic marker of cancer progression. For this purpose, a series of 90 lung adenocarcinomas and 10 uninvolved lung samples were examined for quantitative differences in protein expression using two-dimensional polyacrylamide gel electrophoresis. The β subunit of the mitochondrial H^+-ATP synthase (β-F1-ATPase) and heat shock protein 60 (Hsp 60), and the glycolytic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used to define the bioenergetic cellular (BEC) index, were identified using mass spectrometry and specific antibodies. Correlations of the expression level of the protein markers and of the BEC index were established with the clinicopathological information of the tumors and the follow-up data of the patients. The expression of β-F1-ATPase is significantly reduced in lung adenocarcinomas in the absence of significant changes in the expression of Hsp 60 and of a major GAPDH isoform. Cross-validation analysis using the β-F1-ATPase/Hsp 60 ratio and GAPDH expression as predictor variables revealed a classification sensitivity of 97.3%. The β-F1-ATPase/Hsp 60 ratio is significantly higher in well differentiated and bronchioloalveolar tumors than in moderate or poorly differentiated and in bronchial-derived tumors. The BEC index of T1 tumors was significantly higher than that of T2 tumors. Likewise, stage IA tumors had a higher BEC index than stage IB tumors. Kaplan–Meier survival analysis using the BEC index as predictor of survival revealed that within tumors of the same size or stage I or with no lymph node metastasis (N0) the patients bearing 'low' BEC index tumors had a significant worse prognosis. We conclude that the bioenergetic signature of lung adenocarcinomas is altered, further providing a relevant marker for the diagnosis and classification of lung adenocarcinomas, and for the prognosis of lung cancer patients.

Abbreviations: BEC index, bioenergetic cellular index; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; β-F1-ATPase, β-subunit of the mitochondrial H^+-ATP synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hsp 60, heat shock protein 60

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Introduction

Lung cancer is the most common cause of cancer-related mortality in developed countries. Although the histopathological appearance of lung cancers is very heterogeneous the adenocarcinomas are the most frequently observed non-small cell lung cancer in patients (1). The anatomopathologic extent of the disease (primary Tumor, regional lymph Nodes and distant Metastasis; TNM) is the current classification used to predict the survival of cancer patients (2,3). The recent development of large scale genomic (4–6) and proteomic (7,8) techniques is allowing the analysis of the expression pattern of the genes and proteins that are associated with the phenotype of a particular type of tumor, providing its so-called cancer signature (9,10). One of the main purposes of these studies is to find the molecular markers that could help in the early diagnosis of cancer and that could further predict the prognosis of the patients. In addition, these approaches can unveil previously uncharacterized molecular targets that will aid in the development of new treatment strategies for cancer patients.

Mitochondrial oxidative phosphorylation is the bioenergetic pathway of aerobic cells responsible for the synthesis of most cellular ATP requirements (11,12). The H^+-ATP synthase is the enzyme complex of the inner mitochondrial membrane that carries out the synthesis of ATP. It has been reported that both the activity of oxidative phosphorylation (13,14) and the subunits of the H^+-ATP synthase (15) are required for the execution of programmed cell death. An alteration in the execution of apoptosis is one of the hallmarks of the cancerous cell contributing to the growth of the tumor and cancer progression (16,17). Recently, a decreased expression of the catalytic subunit of the H^+-ATP synthase (β-F1-ATPase), a rate-limiting component of the activity of oxidative phosphorylation, has been reported in human carcinomas of the liver, kidney and colon (18). The finding that an altered bioenergetic phenotype of mitochondria is intimately associated with carcinogenesis suggested that the onset and progression of cancer in these tissues might be due, among other factors, to the impaired contribution of oxidative phosphorylation to the execution of programmed cell death (18).

Interestingly, it was also observed that glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a protein marker of the glycolytic pathway, was concurrently up regulated in kidney and colon cancer (18). These findings suggested that the impairment of the bioenergetic function of mitochondria in the cancer cell is concurrent with the up-regulation of the glycolytic pathway, as originally suggested by Otto Warburg (19). Therefore, we reasoned that the metabolic phenotype of the cell, more specifically its bioenergetic phenotype, could provide a marker for the analysis of cancer progression. For this purpose we developed a proteomic-based bioenergetic cellular index (BEC index) of cellular status that expresses the bioenergetic activity of mitochondria relative to the

glycolytic potential of the cell (18). In tumors of the kidney and colon we showed a large depression of the BEC index when compared with the non-cancerous tissue (18). We further suggested that the BEC index could provide a useful marker for the prognosis of cancer patients. In this regard, we showed that both the expression level of the β-F1-ATPase, and of the bioenergetic signature, had prognostic value in assessing the clinical outcome of patients with Dukes' stage B2 ($T_3N_0M_0$) colorectal carcinomas (18).

In this study we have analyzed the bioenergetic signature of 90 lung adenocarcinomas and 10 uninvolved lung samples using two-dimensional (2D)-PAGE. Quantitative measures of mitochondrial β-F1-ATPase, heat shock protein 60 (Hsp 60) and of the glycolytic GAPDH were obtained following identification of the proteins using mass spectrometry and confirmation with specific antibodies. Correlations in the expression level of the markers with the clinicopathological classification of the tumors were further established. The results obtained reveal that the expression level of the catalytic subunit of the H^+-ATP synthase (β-F1-ATPase) is significantly reduced in lung adenocarcinomas, strongly suggesting that the alteration of the bioenergetic function of mitochondria is also a feature of lung cancer. Overall, the results indicate that proteins that define the metabolic phenotype of the cell, as assessed by the BEC index, provide markers of clinicopathological relevance for the classification of lung adenocarcinomas and the prognosis of lung cancer patients.

Materials and methods

Tissue specimens and preparation

All lung tumors and adjacent normal lung tissue were obtained at the time of surgery at the University of Michigan Hospital from May 1991 to July 2000. Patient consent was received, and the Institutional Review Board approved the project. Patients' medical records were reviewed, and identifiers coded to protect patient confidentiality. Sixty-two stage I lung adenocarcinomas, 28 stage III lung adenocarcinomas and 10 uninvolved lung tissue samples were examined. Processing of the samples has been described previously in detail (8). In brief, hematoxylin-stained cryostat sections (5 μm), prepared from tumor pieces to be used for protein isolation, were evaluated by a pathologist as well as compared with H&E-stained sections made from paraffin blocks of the same tumors. The same selected region of the tumor was used for protein isolation. Lung and tumor tissue (~50–70 mg wet weight) were homogenized and solubilized in a buffer containing 9.5 M urea, 2% Nonidet P-40, 2% ampholines (pI 3.5–10, Pharmacia/LKB, Piscataway, NJ), 2% β-mercaptoethanol and 10 mM phenylmethylsulfonyl fluoride. The insoluble material was removed by centrifugation at 16 000 *g* for 5 min at room temperature. Protein concentration of the extracts was determined using a colorimetric protein assay (Bio-Rad, Hercules, CA). Extracts were stored at −80°C until used. Specimens were excluded if there was: (i) unclear or mixed histology (e.g. adenosquamous); (ii) potential metastatic origin as indicated by previous tumor history; and (iii) the patient had experienced chemotherapy or radiotherapy. In addition, the tumors that were utilized contained at least 70% tumor cellularity and the regions of the tumors were specifically chosen that did not contain significant areas of fibrosis, inflammation, lymphocytes or necrosis following the criteria established by the pathologist. We estimate that in no instances did these non-tumor cells contribute >10% of the total cellular protein for the tumors analyzed. Any tumors that contained such areas were rejected from further analysis. A total of 90 lung adenocarcinoma biopsies were processed by 2D-gels. Tumors were histopathologically divided into two categories: bronchial-derived, if they exhibited invasive features with architectural destruction, or bronchioloalveolar, if they exhibited preservation of the lung architecture.

2D-PAGE and protein quantification

Analytical 2D-PAGE was performed as described previously (8). After separation, the protein spots were visualized by a photochemical silver-based staining technique. Each gel was scanned using a Kodak CCD camera. Spot detection was accomplished using Bio Image Visage System software (Bioimage, Ann Arbor, MI). Each gel generated 1600–2200 detectable spots, and 820 spots were selected for quantitative measurement. The integrated intensity of each spot was calculated as the measured absorbance units multiplied by square millimeters. Each gel's protein spots were matched to the 820 spots on a 'master' gel allowing identification of identical polypeptides between each gel. A total of 250 spots were chosen as ubiquitously expressed reference spots to allow adjustment for subtle variation in protein loading and gel staining. Each of the 820 spots was mathematically adjusted in relation to the reference spots (20,21). Identification of individual protein spots was performed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) in the Perseptive Voyager Biospectrometry Workstation (PerSeptive Biosystem, Framingham, MA) as described previously (8).

The β-F1-ATPase/Hsp 60 ratio and the BEC index (β-F1-ATPase/Hsp 60/GAPDH ratio) were calculated from intra-gel values of the integrated intensity of the protein spots providing in this way a normalized expression level of β-F1-ATPase that is not affected by the processing of the samples.

2D-western blotting

Protein extracts of A549 lung adenocarcinoma cells were run on 2D-gels using the identical conditions as used for the analytical 2D-gels. The separated proteins were transferred onto polyvinylidene fluoride membranes and incubated for 2 h at room temperature with a blocking buffer consisting of TBST (Tris-buffered saline, 0.01% Tween 20) and 5% non-fat dry milk. Individual membranes were washed and incubated with rabbit anti-β-F1-ATPase at a dilution of 1:20 000 (18); mouse monoclonal anti-Hsp 60 (SPA 807, Stressgene, Victoria, Canada) at a dilution of 1:2000 and mouse monoclonal anti-GAPDH at a dilution of 1:10 000 (Abcam, Cambridge, UK) for 1 h at room temperature. After additional washes with TBST, the membranes were incubated with a secondary antibody conjugated with horseradish peroxidase at a 1:5000 dilution for 1 h, further washed, and then incubated 1 min with enhanced chemiluminescence (Pierce, Rockford, IL) prior to exposure to X-ray film.

Statistical analysis

Kruskal–Wallis tests (22) were used to identify differences in the median expression level of the markers between the groups compared. Similar results were obtained using the *t*-test.

The Fisher's linear discriminant function (23) on non-blinded specimens was used to assign the biopsy of the patients to one of two considered classes, normal or tumor. The β-F1-ATPase/Hsp60 ratio and expression level of GAPDH were used as discriminant variables. The actual error rate, or misclassification rate, was estimated by the Lachenbruch's 'holdout' procedure (24), also referred to as cross-validation. It consisted in the omission of one observation, development of the classification function based on the remaining observations and classification of the holdout observation. Repeating this procedure for each observation allowed the estimation of the misclassification rate.

To determine the association between the expression levels of the markers with survival times the median value of the BEC index was used as cut point to define 'high' and 'low' risk groups. Kaplan–Meier survival plots and log rank tests were used (25).

Results

Protein samples from 90 lung adenocarcinomas and 10 uninvolved lung samples were fractionated on 2D-gels (Figure 1a). The mitochondrial β-F1-ATPase (spot 0424, MW 50.9, pI 4.6) and Hsp 60 (spot 0338, MW 57.5, pI 4.9) proteins, as well as the glycolytic GAPDH (spot 0855, MW 35.2, pI 7.5), were identified using mass spectrometry and specific antibodies (Figure 1a). Quantitative measures for the individual protein spots were obtained and the mean expression level of each marker in normal and tumor tissue calculated (Figure 1b). Because of technical limitations the accurate quantification of a particular protein was not possible in some of the processed samples. Overall, we did not obtain the values for β-F1-ATPase in one patient, Hsp 60 in seven patients and GAPDH in 15 patients. The expression level of β-F1-ATPase is significantly lower in lung cancer than in the normal lung (Figure 1b). Interestingly, the change in the expression level of the mitochondrial β-F1-ATPase occurred in the absence of changes for the expression level of the mitochondrial Hsp 60 (Figure 1b), as assessed both by the sum of the integrated

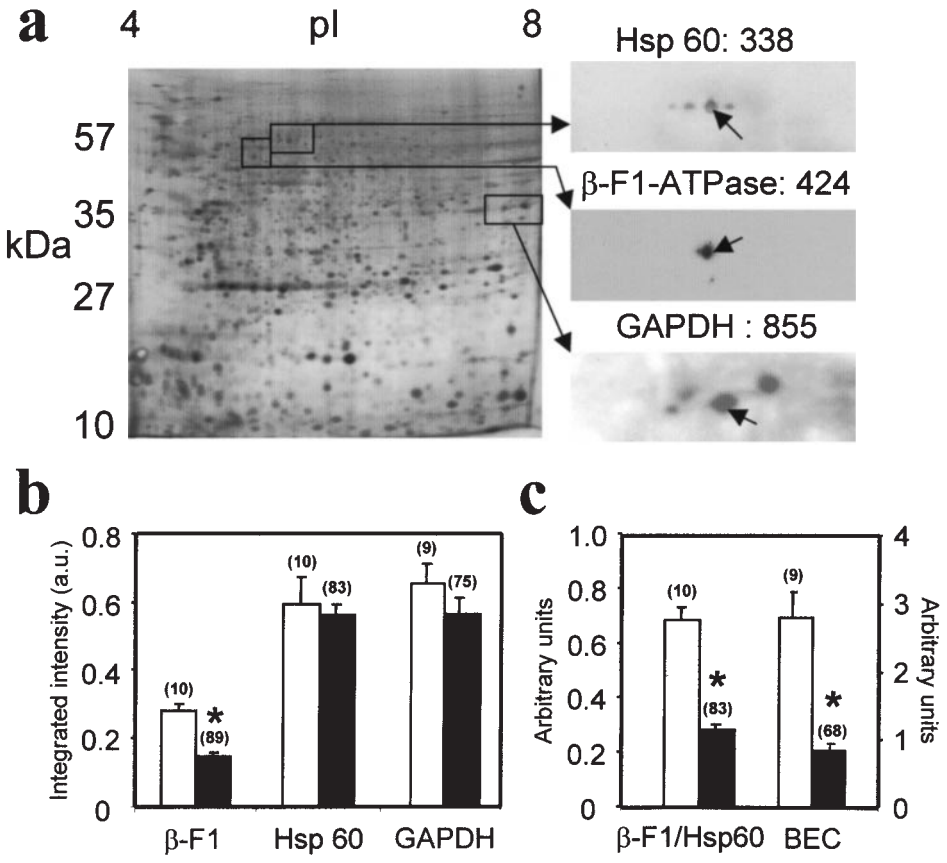

Fig. 1. (**a**) 2D-PAGE separation of proteins of a stage I lung adenocarcinoma identified by silver staining. The proteins are separated by isoelectric point (pI) in the first dimension and by molecular mass (kDa) in the second dimension. The location of the Hsp 60, β-F1-ATPase and GAPDH proteins identified by 2D-gel western blotting are shown (arrows). Protein samples from 90 lung adenocarcinomas and 10 uninvolved lung samples were fractionated on 2D-gels. (**b**) Normalized expression level of the proteins (mean ± SEM) in normal lung (open bars) and in lung adenocarcinomas (closed bars). The number of data is indicated in parentheses. (**c**) Intragel determined β-F1-ATPase/Hsp 60 ratios and BEC indexes (mean ± SEM) in normal (open bars) and adenocarcinomas (closed bars) of the lung. The number of data is indicated in parentheses. $^{*}P < 0.001$ when compared with normal.

intensity of Hsp 60 immunoreactive spots (Figure 1a, data not shown) or by the most abundant (>70%) isoform of the Hsp 60 protein (spot 0338) (Figure 1b).

Isoforms of GAPDH have a basic pI mapping on the edge of the 2D-gels (Figure 1a, pH 8). This situation compromised the accurate quantification of the GAPDH protein spots in some of the processed samples. However, it was found that the expression level of GAPDH (spot 855) that was accurately determined in ~84% of the tumors did not show significant differences between normal and tumor biopsies (Figure 1b). It should be noted that an additional GAPDH isoform (spot 0861, MW 35.2, pI 7.6), mapping on the very edge of the gels, was detected in ~60% of the processed samples (see GAPDH in Figure 1a, the spot to the right hand side of 0855). The 0861 GAPDH isoform showed a significant increase in tumor biopsies when compared with normal lung biopsies (0.37 ± 0.03 and 0.88 ± 0.08 a.u., for normal and tumor, respectively, $P < 0.05$). However, this GAPDH isoform (spot 861) was not included in the below described studies because a large fraction of the tumors analyzed did not have a value for this parameter.

Consistent with the above findings the intra-gel determination of the β-F1-ATPase/Hsp 60 ratio and of the BEC index (β-F1-ATPase/Hsp 60/GAPDH), both providing a direct normalized value of the expression level of β-F1-ATPase between the samples, also showed a marked reduction in lung adenocarcinomas when compared with the normal lung (Figure 1c). These results indicate that the alteration of the bioenergetic signature of mitochondria is associated with carcinogenesis in the lung, as was found previously in kidney and colon cancer (18).

In order to assess the robustness of metabolic marker proteins for cancer diagnosis the Fisher linear discriminant analysis (23) on non-blinded specimens was applied using as predictor variables the mitochondrial bioenergetic competence (β-F1-ATPase/Hsp 60 ratio) and the expression level of the glycolytic GAPDH protein. Using cross-validation (24), it was observed that the overall correct classification of the biopsies was 91.4%, with a sensitivity of 97.3% for the tumor biopsies. The same analysis using the BEC index as a predictor variable showed an overall correct classification of the biopsies of 92.2% with a sensitivity of 92.6%.

Correlations between the expression level of the markers, the β-F1-ATPase/Hsp 60 ratio and of the BEC index with the clinicopathological information of the tumors was carried out (Figure 2). The β-F1-ATPase/Hsp 60 ratio was significantly different according to the histopathological classification of the tumors (Figure 2a). Those tumors exhibiting an invasive feature of the lung architecture, and classified as bronchial-derived, showed a β-F1-ATPase/Hsp 60 ratio lower than that exhibited by tumors of non-invasive features and classified as bronchioloalveolar carcinomas. Similarly, the expression level

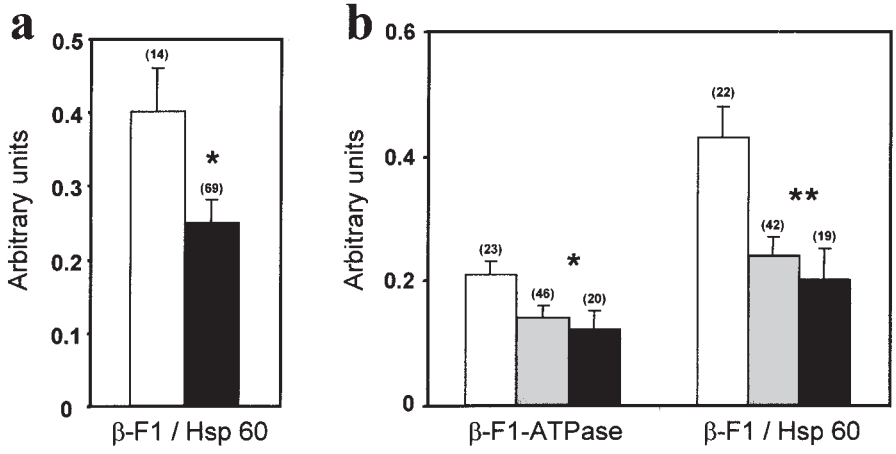

Fig. 2. (**a**) β-F1-ATPase/Hsp 60 ratio in 14 bronchioloalveolar (open bar) and 69 bronchial-derived (closed bar) lung adenocarcinomas. The results shown are the mean ± SEM. $^{*}P < 0.041$ when compared with bronchioloalveolar. (**b**) Expression level of β-F1-ATPase and β-F1-ATPase/Hsp 60 ratio in 23 well (open bars), 46 moderate (gray bars) and 20 poorly (closed bars) differentiated lung adenocarcinomas. The results shown are the mean ± SEM. The number of data is indicated in parentheses. $^{*}P < 0.01$ and $^{**}P < 0.003$ when comparing the moderate and poorly differentiated to well-differentiated adenocarcinomas.

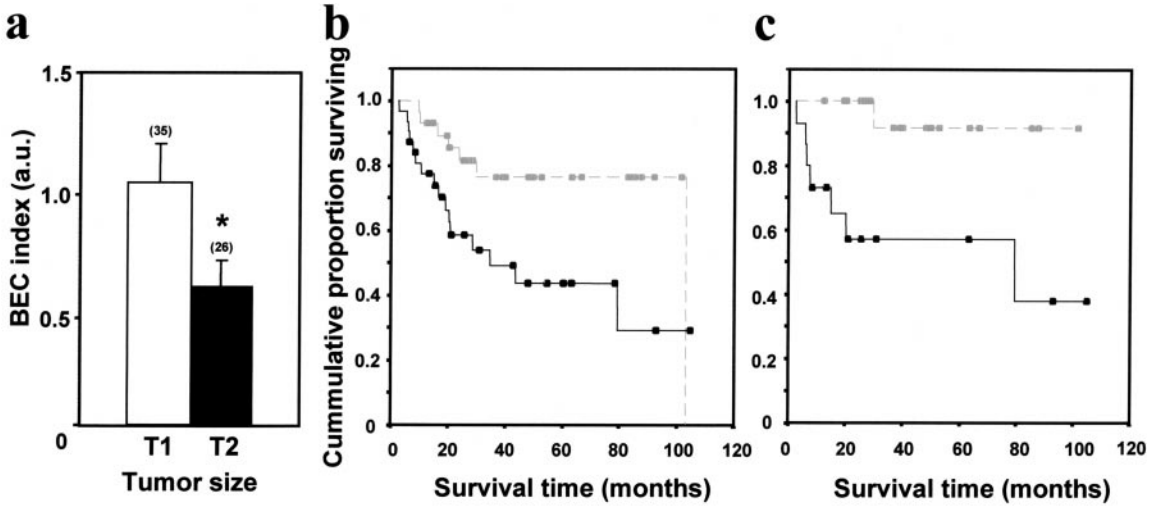

Fig. 3. (**a**) The BEC index in 35 T1 (<3 cm) (open bar) and 26 T2 (>3 cm) (closed bar) size lung adenocarcinomas. The results shown are the mean ± SEM. $^{*}P < 0.036$ when compared with T1 size tumors. (**b**) Kaplan–Meier survival analysis for high (gray curve) versus low (black curve) BEC index in 61 T1 + T2 lung adenocarcinomas. Survival is plotted in the *x* axis versus the BEC index of the tumors. High- and low-BEC index groups were defined by the median BEC index value (0.62) of the study population. The two groups show a significant difference with regard to survival, in that patients in the high-BEC group had a survival advantage ($P = 0.0189$). (**c**) Kaplan–Meier survival analysis for high (gray curve) versus low (black curve) BEC index in 35 T1 lung adenocarcinomas. High- and low-BEC index groups were defined by the median BEC index value. Patients in the high BEC index group had a significant survival advantage ($P = 0.0044$).

of β-F1-ATPase, as well as of the β-F1-ATPase/Hsp 60 ratio, also correlated significantly with the degree of cellular differentiation of the tumors (Figure 2b), showing a progressive decline in these parameters from well differentiated to poorly differentiated tumors.

The BEC index provided significant differences when the size of the tumors was considered (Figure 3a). Larger tumors (T2, >3 cm) had lower BEC indexes than smaller ones (T1, <3 cm). Using Kaplan–Meier survival analysis and the BEC index as predictor of survival it was found that within all the tumors analyzed (T1 + T2) those with a low BEC index correlated with a reduced patient survival (Figure 3b). The median value of the BEC index (0.62) was used to stratify patients into 'high' or 'low' expression groups. Patients in the 'low' expression group had a higher mortality ($P = 0.0189$). When the survival analysis was applied only to patients with tumors of the same size (T1), using the median value of the BEC index for the stratification of the patients, a more significant difference in survival was observed between the patients bearing tumors with a high- and a low-BEC index ($P = 0.0044$, Figure 3c).

Likewise, the BEC index significantly correlated with tumor staging (Figure 4a), showing that stage IA tumors had a 2.5-fold higher value of the BEC index than stage IB tumors. Kaplan–Meier analysis of stage I adenocarcinomas (stage IA + IB) using the BEC index as a predictor of survival, following the procedure above described, revealed that patients bearing a tumor with a high BEC index (>0.62) had a better prognosis than those patients with a tumor falling into the low BEC-index group ($P = 0.029$, Figure 4b). The BEC index of the tumors was also higher in patients with no regional lymph node metastasis (N0) than in those patients with a positive lymph node metastasis (N1-2) (0.94 ± 0.13 and 0.59 ± 0.14 for negative and positive, respectively) although

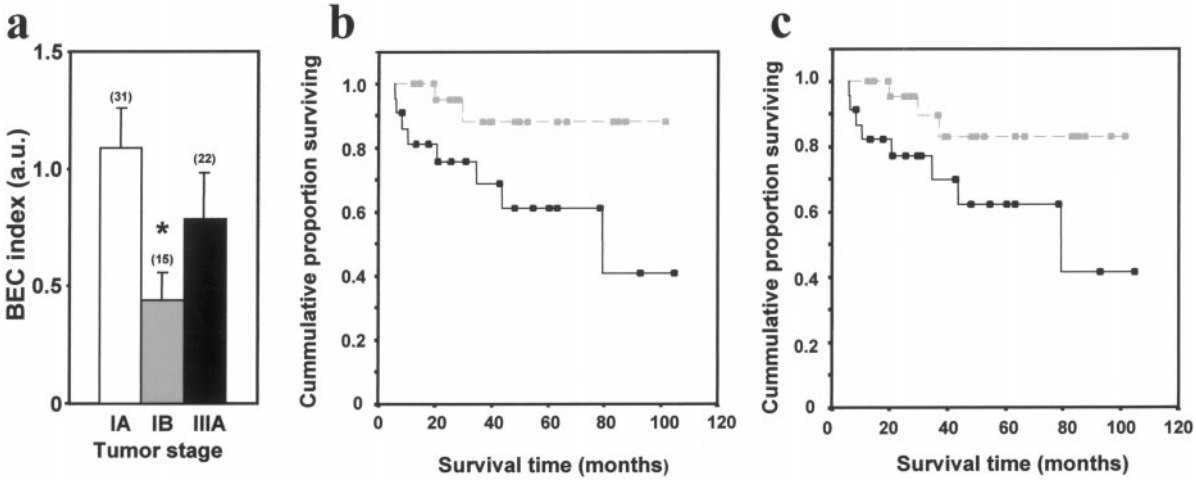

Fig. 4. (**a**) The BEC index in 31 stage IA (open bar), 15 stage IB (gray bar) and 22 stage IIIA (closed bar) lung adenocarcinomas. The results shown are the mean ± SEM. $^{*}P < 0.046$ when compared with stage IA tumors. (**b**) Kaplan–Meier survival analysis for high (gray curve) versus low (black curve) BEC index in 46 stage I (IA + IB) lung adenocarcinomas. Patients in the high BEC index group had a significant advantage in survival ($P = 0.029$). (**c**) Kaplan–Meier survival analysis for 49 patients with negative lymph node metastasis stratified by the BEC index of the lung adenocarcinomas. High- (gray curve) and low- (black curve) BEC index groups were defined by the median BEC index value. Patients in the high BEC index group had a favorable survival advantage ($P = 0.05$).

the difference was not statistically significant ($P = 0.10$). However, Kaplan–Meier analysis of the adenocarcinomas that showed a negative lymph node metastasis using the BEC index as predictor of survival revealed that patients bearing a tumor with a low BEC index (<0.62) had worse prognosis than patients with tumors of a high BEC index ($P = 0.050$, Figure 4c).

Discussion

In this work we provide evidence indicating that the alteration of the bioenergetic signature of mitochondria is a feature of lung adenocarcinomas that provides a quantitative indication that could aid in the histopathological analysis of lung tumor biopsies for the diagnosis, classification and characterization of the differentiation state of the tumors. In addition, the bioenergetic signature of the tumors also provides a molecular marker that is able to discriminate the tumors that have a worse prognosis for the patients. To the extent of our knowledge this is the first time that the expression level of metabolic markers allows us to distinguish between normal and tumor tissue with an observed sensitivity higher than 97%. This figure is higher than that obtained for the most widely used diagnostic markers (cytokeratins, thyroid transcription factor-1 and mesothelial cell markers) of lung cancer. It is also above the figures obtained for prognostic markers in lung adenocarcinomas, such as K-*ras* (~35%), epidermal growth factor receptor (~50%), loss of retinoblastoma staining (~32%) and p53 over-expression (~36%) (26,27).

The expression level of a protein marker does not necessarily reflect the cellular activity of the protein. However, the β-subunit of the H^{+}-ATP synthase is the only catalytic subunit of the complex (11) and thus a bottleneck component in the synthesis of ATP by oxidative phosphorylation. The alteration of the bioenergetic phenotype of mitochondria has also been observed in liver, kidney and colon carcinomas (18). Therefore, we suggest that the bioenergetic signature is indicative of an impairment of the bioenergetic function of mitochondria in the cancer cell and perhaps a required metabolic condition for the development and progression of cancer. It would be interesting to address in future work whether the alteration of the bioenergetic signature in lung adenocarcinomas is accompanied by a parallel down-regulation of other protein complexes involved in mitochondrial energy transduction.

Although the expression of the glycolytic GAPDH isoform used in this study to calculate the BEC index is not significantly affected in lung adenocarcinomas, we do observe an increase in the expression of another GAPDH isoform (spot 0861) in a representative fraction (~60%) of the tumors analyzed. This last finding is consistent with our previous observation in these lung adenocarcinomas that indicate that GAPDH mRNA levels are increased relative to the normal lung (6) and with the increased expression of GAPDH that has been observed in kidney and colon cancer (18). In addition, other glycolytic proteins have been shown to increase in lung cancer and pointed out the relevance of glycolytic markers in lung cancer studies (8,28). For instance, a recent report showed an increase of an isoform of triosephosphate isomerase that correlated with tumor stage in lung adenocarcinomas (8). Therefore, it appears that lung cancer also fits the Warburg hypothesis on the metabolic condition of the cancer cell (19,29) in the sense that the depression of the bioenergetic phenotype of mitochondria is concurrent with the up-regulation of several glycolytic markers.

There is a general consensus that other prognostic factors, different to those included in the TNM-stages system, are required to improve the accuracy in the management of lung cancer patients (26,30). The results reported in this work indicate that the BEC index, that provides a normalized expression value of the mitochondrial bioenergetic competence, affords a convenient molecular marker for the prognosis of the patients. In fact, the BEC index provides a quantitative estimate that allows the discrimination of those tumors that falling within the same group of clinical stage (size, stage and lymph node metastasis) by the TNM-system have a poorer prognosis. In this regard, we suggest that the determination of the bioenergetic signature of the tumor will benefit clinical practice because it can be anticipated that the BEC index will also provide a predictive marker of the response of the tumor to

chemo- and/or radiotherapy, thus helping to define the treatment that should be applied to the patients and/or eventually spare an unnecessary treatment to a subset of them. Remarkably, the determination of the bioenergetic signature of the tumors may not require a sophisticated proteomic approach since it can be assessed in most laboratories by common immunological techniques such as conventional western blots and immunohistochemistry (18).

Mitochondrial oxidative phosphorylation (13,14) and the H^+-ATP synthase (15) are required for the execution of programmed cell death. An impaired bioenergetic competence of mitochondria as assessed by the bioenergetic signature is a feature of liver, kidney, colon (18) and lung cancer (this study). In those cases where a larger cohort of tumors has been studied, such as in colon (18) and lung cancer (this study), the bioenergetic signature has been shown to be intimately associated with the progression of the disease. Although the contribution of mitochondrial bioenergetics to the overall metabolic accomplishment of the cell could vary between different cell types, and so the contribution of the H^+-ATP synthase, our findings indicate that further efforts are required to address the implication of mitochondrial bioenergetics in the development and progression of cancer. Specifically, on the functional role that is played by the H^+-ATP synthase in apoptosis (15), by analyzing the mechanistic contribution of this protein complex in tumor suppression.

The regulation of gene expression of the subunits encoding components of the H^+-ATP synthase is very complex affecting both transcriptional and post-transcriptional levels (31–35). In this regard, specific translation control mechanisms regulate the expression of β-F1-ATPase in mammalian cells during development (31,32,36,37) and in oncogenesis (38,39). Interestingly, the expression of β-F1-ATPase mRNA in lung cancer is up regulated when compared with the normal lung (40). Although the mechanisms that promote a decreased expression of β-F1-ATPase in lung cancer are at present time largely unknown, it is very relevant to point out that in lung adenocarcinomas the expression level of a counterpart subunit of this protein complex (δ-F1-ATPase) is increased (8). Therefore, it appears that carcinogenesis in the lung is specifically affecting the expression of the β-subunit. These findings therefore reinforce the well documented idea that mRNA abundance cannot predict overall protein expression levels (41) and that post-transcriptional regulatory mechanisms that affect key components of the energy transduction pathway are essential for our understanding of carcinogenesis in the lung.

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References

1. Fry,W.A., Phillips,J.L. and Menck,H.R. (1999) Ten-year survey of lung cancer treatment and survival in hospitals in the United States: a national cancer data base report. *Cancer*, **86**, 1867–1876.
2. Mountain,C.F. (2000) The international system for staging lung cancer. *Semin. Surg. Oncol.*, **18**, 106–115.
3. Mountain,C.F. (2002) Staging classification of lung cancer. A critical evaluation. *Clin. Chest Med.*, **23**, 103–121.
4. Ramaswamy,S. and Golub,T.R. (2002) DNA microarrays in clinical oncology. *J. Clin. Oncol.*, **20**, 1932–1941.
5. Petricoin,E.F., Zoon,K.C., Kohn,E.C., Barrett,J.C. and Liotta,L.A. (2002) Clinical proteomics: translating benchside promise into bedside reality. *Nature Rev. Drug Discov.*, **1**, 683–695.
6. Beer,D.G., Kardia,S.L., Huang,C.C. *et al.* (2002) Gene-expression profiles predict survival of patients with lung adenocarcinoma. *Nature Med.*, **8**, 816–824.
7. Wulfkuhle,J.D., Liotta,L.A. and Petricoin,E.F. (2003) Proteomic applications for the early detection of cancer. *Nature Rev. Cancer*, **3**, 267–275.
8. Chen,G., Gharib,T.G., Huang,C.C. *et al.* (2002) Proteomic analysis of lung adenocarcinoma: identification of a highly expressed set of proteins in tumors. *Clin. Cancer Res.*, **8**, 2298–2305.
9. Ramaswamy,S., Ross,K.N., Lander,E.S. and Golub,T.R. (2003) A molecular signature of metastasis in primary solid tumors. *Nature Genet.*, **33**, 49–54.
10. Liotta,L.A. and Kohn,E.C. (2003) Cancer's deadly signature. *Nature Genet.*, **33**, 10–11.
11. Boyer,P.D. (1997) The ATP synthase. A splendid molecular machine. *Annu. Rev. Biochem.*, **66**, 717–749.
12. Yoshida,M., Muneyuki,E. and Hisabori,T. (2001) ATP synthase—a marvellous rotary engine of the cell. *Nature Rev. Mol. Cell Biol.*, **2**, 669–677.
13. 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.
14. 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
15. 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.
16. Reed,J.C. (1999) Mechanisms of apoptosis avoidance in cancer. *Curr. Opin. Oncol.*, **11**, 68–75.
17. Hanahan,D. and Weinberg,R.A. (2000) The hallmarks of cancer. *Cell*, **100**, 57–70.
18. Cuezva,J.M., Krajewska,M., López de Heredia,M., Krajewski,S., Santamaría,G., Kim,H., Zapata,J.M., Marusawa,H., Chamorro,M. and Reed,J.C. (2002) The bioenergetic signature of cancer: a marker of tumor progression. *Cancer Res.*, **62**, 6674–6681.
19. Warburg,O. (1930) *Metabolism of Tumors*. Arnold Constable, London.
20. Ali,I., Chan,Y., Kuick,R., Teichroew,D. and Hanash,S.M. (1991) Implementation of a two-dimensional electrophoresis-related laboratory information processing system: database aspects. *Electrophoresis*, **12**, 747–761.
21. Kuick,R.D., Skolnick,M.M., Hanash,S.M. and Neel,J.V. (1991) A two-dimensional electrophoresis-related laboratory information processing system: spot matching. *Electrophoresis*, **12**, 736–746.
22. Daniel,W.W. (1990) *Applied Nonparametric Statistics*. PSW-KENT Publishing Company, Boston.
23. Johnson,R.A. and Wichern,D.W. (1992) *Applied Multivariate Statistical Analysis*. Prentice-Hall, New Jersey.
24. Lachenbruch,P.A. and Mickey,M.R. (1968) Estimation of error rates in discriminant analysis. *Technometrics*, **10**, 1–11.
25. Cox,D.R. and Oakes,D. (1988) *Analysis of Survival Data*. Chapman and Hall Ltd, London.
26. Iyengar,P. and Tsao,M.S. (2002) Clinical relevance of molecular markers in lung cancer. *Surg. Oncol.*, **11**, 167–179.
27. Mao,L. (2002) Recent advances in the molecular diagnosis of lung cancer. *Oncogene*, **21**, 6960–6969.
28. Wu,J.T. (1999) Review of circulating tumor markers: from enzyme, carcinoembryonic protein to oncogene and suppressor gene. *Ann. Clin. Lab. Sci.*, **29**, 106–111.
29. Warburg,O. (1956) On the origin of cancer cells. *Science*, **123**, 309–314.
30. Lopez-Encuentra,A., Bulzebruck,H., Feinstein,A.R., Motta,G., Mountain,C.F., Naruke,T., Sanchez,J.M., Tsuchiya,R. and Wittekind,C. (2000) Tumor staging and classification in lung cancer. *Lung Cancer*, **29**, 79–83.
31. Izquierdo,J.M., Ricart,J., Ostronoff,L.K., Egea,G. and Cuezva,J.M. (1995) Changing patterns of transcriptional and post-transcriptional control of beta-F1-ATPase gene expression during mitochondrial biogenesis in liver. *J. Biol. Chem.*, **270**, 10342–10350.

32. 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.
33. Hayakawa,M., Sakashita,E., Ueno,E., Tominaga,S., Hamamoto,T., Kagawa,Y. and Endo,H. (2002) Muscle-specific exonic splicing silencer for exon exclusion in human ATP synthase gamma-subunit pre-mRNA. *J. Biol. Chem.*, **277**, 6974–6984.
34. Jordan,E.M., Worley,T. and Breen,G.A. (2003) Transcriptional regulation of the nuclear gene encoding the alpha-subunit of the mammalian mitochondrial F1F0 ATP synthase complex: role for the orphan nuclear receptor, COUP-TFII/ARP-1. *Biochemistry*, **42**, 2656–2663.
35. Jin,Y., Suzuki,H., Maegawa,S., Endo,H., Sugano,S., Hashimoto,K., Yasuda,K. and Inoue,K. (2003) A vertebrate RNA-binding protein Fox-1 regulates tissue-specific splicing via the pentanucleotide GCAUG. *EMBO J.*, **22**, 905–912.
36. Luis,A.M., Izquierdo,J.M., Ostronoff,L.K., Salinas,M., Santarén,J.F. and Cuezva,J.M. (1993) Translational regulation of mitochondrial differentiation in neonatal rat liver. Specific increase in the translational efficiency of the nuclear-encoded mitochondrial beta-F1-ATPase mRNA. *J. Biol. Chem.*, **268**, 1868–1875.
37. Ricart,J., Izquierdo,J.M., Di Liegro,C.M. and Cuezva,J.M. (2002) The assembly of the ribonucleoprotein complex containing the mRNA of the β-subunit of the mitochondrial H^+-ATP synthase requires the participation of two distal *cis*-acting elements and a complex set of cellular *trans*-acting proteins. *Biochem. J.*, **365**, 417–428.
38. 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.
39. López de Heredia,M., Izquierdo,J.M. and Cuezva,J.M. (2000) A conserved mechanism for controlling the translation of beta-F1-ATPase mRNA between the fetal liver and cancer cells. *J. Biol. Chem.*, **275**, 7430–7437.
40. Lal,A., Lash,A.E., Altschul,S.F. *et al.* (1999) A public database for gene expression in human cancers. *Cancer Res.*, **59**, 5403–5407.
41. Chen,G., Gharib,T.G., Huang,C.C. *et al.* (2002) Discordant protein and mRNA expression in lung adenocarcinomas. *Mol. Cell Proteomics*, **1**, 304–313.

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