ACCELERATED PUBLICATION

Alteration of the bioenergetic phenotype of mitochondria is a hallmark of breast, gastric, lung and oesophageal cancer

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Recent findings indicate that the expression of the β-catalytic subunit of the mitochondrial H^+-ATP synthase (β-F_1-ATPase) is depressed in liver, kidney and colon carcinomas, providing further a bioenergetic signature of cancer that is associated with patient survival. In the present study, we performed an analysis of mitochondrial and glycolytic protein markers in breast, gastric and prostate adenocarcinomas, and in squamous oesophageal and lung carcinomas. The expression of mitochondrial and glycolytic markers varied significantly in these carcinomas, when compared with paired normal tissues, with the exception of prostate cancer. Overall, the relative expression of β-F_1-ATPase was significantly reduced in breast and gastric adenocarcinomas, as well as in squamous oesophageal and lung carcinomas, strongly suggesting that alteration of the bioenergetic function of mitochondria is a hallmark of these types of cancer.

Key words: cancer, glycolysis, H^+-ATP synthase, mitochondria.

INTRODUCTION

The H^+-ATP synthase is the mitochondrial protein complex responsible for harnessing cellular ATP [1]. Efficient execution of programmed cell death also requires the activity and molecular components of the H^+-ATP synthase [2–4]. Recently, decreased expression of the β-catalytic subunit of the H^+-ATP synthase (β-F_1-ATPase or βF_1 in ratios) has been demonstrated in liver, kidney and colon carcinomas [5]. These findings provide molecular evidence that support an altered bioenergetic function of mitochondria in these types of cancer [5], in agreement with the Warburg hypothesis [6]. In this regard, we defined a bioenergetic cellular index (BEC index) that could be used to estimate tumour status [5]. The BEC index takes into consideration the expression level of a bioenergetic marker of mitochondria relative to a cellular glycolytic marker, which can be easily determined in tissue biopsies by immunohistochemical or immunoblotting analysis [5]. The down-regulation of the β-F_1-ATPase protein and assessment of the BEC index in colon carcinomas was shown further to have prognostic value in assessing the clinical outcome of patients with early-stage disease [5].

The possible impairment of the bioenergetic function of mitochondria in other human tumours has not been explored. In the present study, we have examined the expression of β-F_1-ATPase as protein marker of oxidative phosphorylation and of hsp60 (heat-shock protein 60) as marker of structural mitochondrial protein, in combination with two markers of the glycolytic pathway [GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and PK (pyruvate kinase)], in randomly selected biopsies of primary breast, gastric and prostate adenocarcinomas, and in squamous lung and oesophageal carcinomas, as well as in the normal tissue biopsies of the same patients. We show that the expression of β-F_1-ATPase, either alone or in combination with mitochondrial and glycolytic markers (as defined by the BEC index), is significantly diminished in breast, gastric, lung and oesophageal cancers. Overall, the findings suggest that an impaired bioenergetic function of mitochondria is a hallmark of carcinogenesis in these human tissues. Furthermore, we suggest that the bioenergetic signature could provide a convenient tool for the diagnosis of patients bearing breast, lung, gastric and oesophageal cancers.

EXPERIMENTAL

Patient specimens

Frozen tissue sections of 17 μm thickness, obtained from human biopsies of untreated patients with primary ductal invasive breast adenocarcinomas, gastric and prostate adenocarcinomas, and squamous oesophageal and lung carcinomas, were obtained from the Banco de Tejidos y Tumores, IDIBAPS (Instituto de Investigaciones Biomédicas Augusto Pi y Suñer), Hospital Clinic, Barcelona, Spain. The tissue sections of the tumour and normal tissue of each patient were analysed previously by an expert pathologist. All tissue samples used in the present study were anonymized and received in a coded form to protect patient confidentiality under IRB (Internal Review Board) approval.

Protein extraction

Approx. 20 tissue sections were extracted in 300 μl of 50 mM Tris/HCl, pH 8, containing 150 mM NaCl, 0.02 % (w/v) sodium azide, 0.1 % (w/v) SDS, 1 % (v/v) Nonidet P40, 0.5 % (w/v) sodium deoxycholate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml antitripsin, 0.4 mM EDTA, 10 mM NaF and 0.75 mM PMSF at 4 °C for 30 min. After protein extraction, the

Abbreviations used: β-F_1-ATPase, β-subunit of the mitochondrial H^+-ATP synthase; BEC index, bioenergetic cellular index; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hsp60, heat-shock protein 60; PK, pyruvate kinase.

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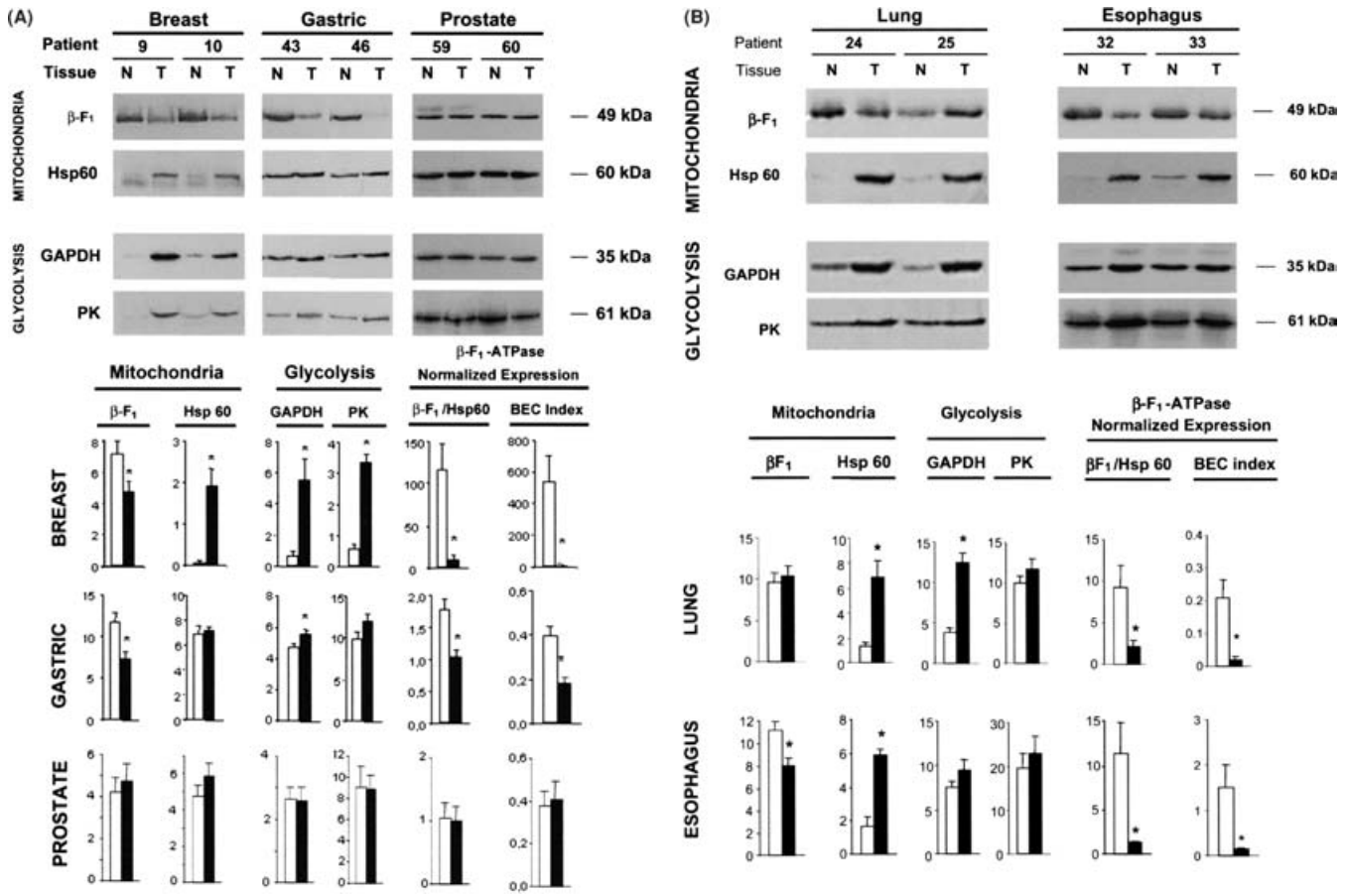

Figure 1 Expression of mitochondrial and glycolytic marker proteins in human tumours

Protein samples from normal (N) and tumour (T) biopsies from the same patient were fractionated by SDS/PAGE (10 % or 15 % gels) and then blotted with the corresponding antibodies. Markers of oxidative phosphorylation (β-F_1-ATPase) and structural function (hsp60) of the mitochondria and of the glycolytic pathway (GAPDH and PK) were studied. The electrophoretic migration (kDa) of each marker is shown on the right-hand side of the Figure. For illustration purposes, the results of two patients are shown for each type of carcinoma. The histograms show the means $\pm$ S.E.M. of the cellular content of each marker as determined by densitometric analysis (arbitrary units) in normal (open bars) and tumour (closed bars) biopsies. The βF_1/hsp60 ratio and BEC index ([βF_1/hsp60]/GAPDH ratio) provide a normalized expression level of β-F_1-ATPase for each sample. $^*P < 0.05$ when compared with normal tissue by Student's t test. (**A**) Breast ($n = 10$), gastric ($n = 10$) and prostate ($n = 9$) adenocarcinomas. (**B**) Squamous lung ($n = 9$) and oesophageal ($n = 6$) carcinomas.

samples were centrifuged (15000 ***g***) 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.

Western blotting

Western blots for paired normal and tumour samples of the same patient were run in the same gel. Samples (15 or 30 μg of protein) were subjected to SDS/PAGE (10 % or 15 % gels) followed by immunoblot analysis [7] using the appropriate dilution of various antisera. The antibodies used in the present study included: rabbit anti-β-F_1-ATPase at 1:20000 dilution [5]; mouse monoclonal anti-hsp60 (SPA 807; Stressgene, Victoria, Canada) at 1:2000 dilution; goat polyclonal anti-(muscle PK) at 1:2000 dilution and mouse monoclonal anti-GAPDH at 1:10000 dilution, both from Abcam (Cambridge, U.K.). Secondary horseradish-peroxidase-conjugated goat anti-rabbit, anti-mouse or rabbit anti-goat antibodies (1:3000 dilution) were used for detection, which was accomplished using an enhanced chemiluminescence detection method (ECL®; Amersham Biosciences). Quantification of the intensity of the immunoreactive bands (arbitrary units) was accomplished using a Kodak DC120 Zoom digital camera and the Kodak 1D Image Analysis Software for Windows. To calculate the normalized expression level of β-F_1-ATPase, the band intensity of β-F_1-ATPase was divided by the band intensity of hsp60 assayed for the same sample and in the same membrane. To calculate the BEC index [5], the aforementioned ratio was normalized relative to the band intensity of GAPDH. Statistical analysis was performed using Student's t test for paired samples.

RESULTS

Breast adenocarcinomas

The analysis of the expression level of mitochondrial and glycolytic marker proteins in ten primary breast ductal invasive adenocarcinomas and in their corresponding paired normal tissue biopsies revealed that the levels of β-F_1-ATPase protein were reduced in seven of the tumours analysed (Figure 1A). In contrast, the expression of mitochondrial hsp60 was increased in all of the tumours analysed (Figure 1A). The glycolytic GAPDH and PK markers were elevated in 80 % and 100 % of the tumours analysed respectively (Figure 1A). The normalized expression

level of β-F_1-ATPase, as assessed by the βF_1/hsp60 ratio, showed a significant reduction in breast adenocarcinomas when compared with normal breast samples (Figure 1A). Likewise, the BEC index ([βF_1/hsp60]/GAPDH ratio) showed that breast cancer samples have a BEC index much lower than that in the normal breast tissue (Figure 1A).

Gastric adenocarcinomas

The analysis of the expression level of β-F_1-ATPase in ten gastric adenocarcinomas and in their corresponding paired normal tissue samples revealed that the marker was significantly reduced in 90% of the tumours analysed (Figure 1A). In contrast, the expression of the mitochondrial hsp60 was not affected in gastric adenocarcinomas when compared with normal tissues (Figure 1A). A slight, marginally significant increase was observed in the expression of GAPDH, whereas PK was not significantly affected in adenocarcinomas of the stomach (Figure 1A). The expression of β-F_1-ATPase relative to that of hsp60 revealed a significant decrease of this ratio in all the tumours when compared with normal tissue (Figure 1A). Consistent with the above findings, the BEC index showed a significant decrease in the adenocarcinomas (Figure 1A).

Prostate adenocarcinomas

No significant differences were observed in the expression level of β-F_1-ATPase and hsp60 in nine prostate adenocarcinomas when compared with their corresponding normal tissue controls (Figure 1A). Likewise, the expression of GAPDH and PK was not significantly affected in prostate cancer (Figure 1A). Therefore the normalized expression levels of β-F_1-ATPase and BEC index were not altered in prostate adenocarcinomas (Figure 1A).

Squamous carcinomas of the lung

The expression of β-F_1-ATPase was not significantly altered in the nine squamous lung carcinomas analysed when compared with normal tissue (Figure 1B). However, the expression of hsp60 was significantly increased in the tumours (Figure 1B). An increased expression of GAPDH was also observed in all the carcinomas when compared with normal samples (Figure 1B). In contrast, the expression of PK was not significantly altered in squamous lung carcinomas (Figure 1B). The expression of β-F_1-ATPase normalized to hsp60 revealed a significant reduction in the tumours when compared with the normal tissue (Figure 1B). Consistent with the above findings, the BEC index revealed a significant decrease in squamous lung carcinomas when compared with normal lung (Figure 1B).

Squamous carcinomas of the oesophagus

Five of the six squamous oesophageal carcinomas analysed showed a significant reduction in the expression of β-F_1-ATPase when compared with its normal paired sample (Figure 1B). Interestingly, the expression of the mitochondrial hsp60 marker was significantly increased in the carcinomas when compared with the normal tissue (Figure 1B). The expression of GAPDH and PK was not significantly altered in oesophageal carcinomas (Figure 1B). Consistent with these findings, both the normalized expression level of β-F_1-ATPase (βF_1/hsp60 ratio) and the BEC-index revealed a significant decrease in the carcinomas (Figure 1B).

DISCUSSION

Large-scale genomic [8,9] and proteomic [10] techniques have allowed the analysis of the expression pattern of the genes and proteins that are associated with the phenotype of a particular type of tumour, providing its so-called 'cancer signature' [11,12]. We have recently shown that the mitochondrial and glycolytic phenotypes of colon carcinomas provide a bioenergetic signature of cancer that has prognostic value in clinical practice [5]. In the present paper, we have analysed the expression of a set of mitochondrial and glycolytic markers in other types of common human carcinomas to explore whether or not the alteration of the bioenergetic signature is a generalized condition of cancer. We show that, with the exception of prostate adenocarcinomas, the bioenergetic signature of cancer, as assessed by the absolute expression level of β-F_1-ATPase (breast, oesophagus and stomach) or by its relative expression when considering other mitochondrial and cellular proteins (lung, breast, oesophagus and stomach), provides a molecular marker of carcinogenesis. Although the expression level of a protein marker does not necessarily reflect the cellular activity of the protein, the findings reported in the present paper, in addition to previous observations in liver, kidney and colon carcinomas [5], strongly suggest that altered bioenergetic function of mitochondria is a hallmark of carcinogenesis, as was hypothesized by Warburg almost 80 years ago [6]. The probable metabolic consequence of this impairment is the increased production of cellular ATP by enhanced rates of glycolysis. In this regard, we observed that, with the exception of prostate and oesophageal cancer, the GAPDH marker of the glycolytic pathway is increased in breast, gastric, lung, kidney and colon tumours (Figures 1A and 1B; and [5]). However, in those tumours where PK has been determined (the present study), we only observed a significant increase in the marker in breast tumours (Figures 1A and 1B). This finding could suggest that, in gastric and lung carcinomas, the relative cellular content and/or the regulation of the activity of PK are sufficient to channel through glycolysis the increase in carbon skeletons supplied by an augmented expression of GAPDH. In this regard, it has been documented that the induction of glycolytic genes by glucose is specific for some genes and not all of those that are induced are activated to the same extent and with the same kinetics [13].

The glycolytic and mitochondrial proteome of the different mammalian cell types differ substantially, both in terms of the relative expression level of the expressed proteins and in the expression of cell-type-specific isoforms, reflecting the relative relevance of the two energy-production pathways and the variable energetic demands of each cell type. In this regard, we observed a variable tissue response in the expression of the structural hsp60 marker of the mitochondria as a result of carcinogenesis. This finding is consistent with the de-regulation by carcinogenesis of the cell-type-specific programmes that control mitochondrial biogenesis and function in different mammalian tissues [14–17]. In this regard, the relative cellular increase in hsp60 expression observed in breast, lung and oesophageal cancer could suggest an activation of the programme of mitochondrial proliferation in these tumours as an attempt to compensate for the restrained cellular production of ATP by oxidative phosphorylation. In contrast, the lack of increase of hsp60 observed in gastric adenocarcinomas (Figure 1A), as well as in kidney and colon cancer [5], suggests that, although the functional differentiation [14,18,19] of the organelle is affected, in these types of cancer, there is no activation of mitochondrial proliferation. As recently discussed, the reduction of bioenergetic, structural and genetic markers of mitochondria observed in rat [20] and human hepatocarcinomas [21] is a paradigm of repression of the mechanisms

that regulate mitochondrial proliferation in the liver. Altogether, it appears that carcinogenesis differentially affects the phenotype of mitochondria in a tissue-specific manner, except in prostate cancer. In any case, we suggest that the BEC index could provide a convenient tool for the diagnosis of cancer patients bearing breast, lung, gastric and oesophageal tumours. The usefulness of the bioenergetic signature as a clinical marker should be explored in the near future in these types of cancer.

The mechanism by which the expression of β-F_1-ATPase is repressed in human cancers is not known. The expression of β-F_1-ATPase mRNA in human tumours of the brain, colon, eye, kidney, liver, lung, lymph nodes, mammary gland, placenta, prostate, skin, testis and uterus is up-regulated ($P < 0.05$) when compared with the normal tissue, as assessed by virtual Northern blotting using publicly available databases [22]. These findings strongly suggest that the control of β-F_1-ATPase expression in cancer is exerted at the level of translation of its mRNA. Indeed, translational control of β-F_1-ATPase expression has been documented in the liver during development [23–25], in rat hepatomas [7] and in the tissue-specific expression of this protein in mammalian tissues [25]. The mechanisms that control β-F_1-ATPase mRNA translation in mammalian cells are complex [25–28]. However, repression of β-F_1-ATPase mRNA translation is associated with increased expression and/or activity of specific β-F_1-ATPase mRNA-binding proteins that are regulated during development [25], in a tissue-specific manner [25] and in oncogenesis [7]. The molecular identification of these binding proteins, as well as the factors that regulate their RNA-binding activity should be targets of future cancer investigations. Likewise, the finding that β-F_1-ATPase expression is repressed in most tumours suggests that further efforts are required to understand the mechanistic contribution of the mitochondrial H^+-ATP synthase in cancer progression.

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