Reverse Phase Protein Microarrays Quantify and Validate the Bioenergetic Signature as Biomarker in Colorectal Cancer.

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Abbreviations:

CRC, colorectal carcinoma; β-F1-ATPase, subunit β of the mitochondrial H^+-ATP synthase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; PK-M2, pyruvate kinase M2; Hsp60, mitochondrial heat schock protein 60.

Summary

The development of high-throughput techniques of profiling for validation of new molecular markers of colorectal cancer (CRC) is required. The *bioenergetic signature*, a protein ratio between the mitochondrial β-F1-ATPase and the glycolytic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), affords a marker of the metabolic activity of the tumor that also contributes to the prognosis and treatment of CRC patients. Herein, we have applied *reverse phase protein microarray* (RPMA) technology to quantify the proteins of energetic metabolism β-F1-ATPase, Hsp60, GAPDH and pyruvate kinase M2 (PK-M2) in paired normal and tumor biopsies of forty CRC patients. Correlations of proteins content and of the *bioenergetic signature* were established with the clinicopathological information of the tumors and the follow-up data of the patients. The metabolic proteome of colon cancer specimens revealed a profound shift towards and enhanced glycolytic phenotype concurrent with a profound alteration of their mitochondria (β-F1-ATPase/Hsp60 ratio). Discriminant analysis using markers of the metabolic signature as predictor variables revealed a classification specificity of 91.7% and a sensitivity of 78.9%. Kaplan-Meier survival analysis showed that the *bioenergetic signature* significantly correlated with overall and disease-free survival of the patients. Markers of energetic metabolism allowed the discrimination of CRC patients into two groups with highly significant overall and disease-free prognosis. Cox regression analysis further supported the significant association of the *bad* metabolic signature of the tumor with worst prognosis. Overall, the alteration of the mitochondrial and glycolytic proteome is a hallmark of CRC. We conclude that the quantification and validation of the *bioenergetic signature* as a relevant biomarker of CRC could contribute in the future handling of these patients.

Introduction

Colorectal cancer (CRC) is a common neoplasia which poses a heavy burden on public health systems (1) with an incidence rate that has increased in the last two decades (2). The development of colorectal cancer from normal epithelial cells to malignant carcinomas is a multistep process that involves the accumulation of mutations in oncogenes and tumor suppressor genes (3). Less than 10% of CRC cases arise from highly-penetrant inherited mutations (4) and genome-wide association studies suggest that spontaneous cases, which represent ~ 70% of CRC cases, might be influenced by predisposing single nucleotide polymorphisms (5). Recent findings also implicate the tumor microenvironment in the progression of CRC (6). Despite the establishment of screening protocols for CRC, tailored therapeutic approaches are required to minimize the impact of the disease (1). At present, KRAS mutation status is the only validated predictive marker for targeted CRC therapy (7). Thus, the development and clinical implementation of new predictive molecular markers are required to aid in the selection of patients likely to respond to therapy and rationalized CRC treatments (7).

The altered energetic metabolism of cancer cells has been proposed as a potential target for cancer treatment (8-11). In the specific case of CRC, the enhanced glycolytic phenotype of the tumor, as assessed by the expression of the glycolytic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is accompanied by a diminished expression of the catalytic subunit of mitochondrial H^+-ATP synthase (β-F1-ATPase) (6, 12), a rate-limiting component required for the synthesis of biological energy. This "protein signature" (β-F1-ATPase/GAPDH ratio), which has been defined as the "*bioenergetic signature*" (12), is likely to contribute to the prognosis and treatment of CRC patients (8). Indeed, the *bioenergetic signature* is significantly down-regulated in colorectal tumors when compared to paired normal tissues (12). Moreover, two

independent studies in large cohorts of CRC patients indicated that low tumor expression of β-F1-ATPase is associated with poor overall and disease-free survival of the patients (12, 13), strongly suggesting that impaired mitochondrial bioenergetics is at the heart of colon cancer progression. Consistent with these findings it has been recently shown at the genomic level that oxidative phosphorylation was the only pathway that had significant association with survival in another large cohort of CRC patients (14). In addition, the *bioenergetic signature* also represents a functional index of metabolic activity because it correlates, both *in vivo* and *in vitro*, with the rate of glucose utilization by tumors and cancer cells (6, 15). Furthermore, the bioenergetic signature also correlates with the potential to execute cell death in response to different chemotherapeutical strategies in diverse cancer cell lines (16-19) and, in the specific case of colon cancer, in cells (20, 21), human tumor xenografts (6, 21) and CRC patients (13).

Reverse phase protein microarrays (RPMA) is a high-throughput proteomic technique that allows the quantification of a given marker in minute amounts of protein from biological specimens (22-25). The application of this technique in oncology is most useful for the search and quantification of biomarkers of survival and of the response to chemotherapy. In an effort to translate the *bioenergetic signature* to the clinics we have previously developed high affinity and specific monoclonal antibodies against proteins of energetic metabolism (26). In the present investigation we have applied RPMA technology to quantify and study the relevance of the bioenergetic signature as a maker of diagnosis and prognosis in a cohort of forty colorectal cancer patients. The results obtained provide, for the first time, the quantification of the proteins of energetic metabolism in normal and tumor tissue of CRC patients, validate the clinical utility of the *bioenergetic signature* as a marker of prognosis and highlight

the potential applicability of the *bioenergetic signature* as a predictive tool to assess therapeutic responses in CRC patients.

Experimental Procedures

Patients and Protein extraction. Frozen tissue sections obtained from surgical specimens of untreated cancer patients with primary colorectal adenocarcinomas were obtained from the Banco de Tejidos y Tumores, Hospital Meixoeiro, Vigo, Spain. Routine histopathological study of all the cases had been previously performed by an experienced pathologist and recorded (27). All tissue samples were anonymized and received in a coded form to protect patient confidentiality. The Institutional Review Board approved the project and samples were provided with informed consent from the patients. For protein extraction, the samples were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Inc) containing protease inhibitors (Roche) in a 1:5 (w/v) ratio, and further freeze-thawed three times in liquid nitrogen. After protein extraction, the samples were centrifuged (15000 *g*) at 4ºC for 30 min. The protein concentration was determined with the Bradford reagent (Bio-Rad, Inc) using BSA as a standard. Aliquots were stored at -80ºC until use.

Expression and Purification of Recombinant Proteins. The pQE expression plasmids encoding the human β-F1-ATPase, Hsp60, GAPDH and PK-M2 proteins with C-terminal histidine and streptavidin tags (26) were used to transform *E. coli* BL21 cells. After induction of protein expression by addition of isopropyl-β-thiogalactopiranoside (1 mM), the cells were resuspended in buffer A (100 mM $NaH_2PO_4 \cdot H_20$, 300 mM NaCl, pH 8.0 supplemented with lysozyme 1 mg/ml). The expressed proteins were purified using either Strep-Tactin or metal ion affinity chromatography Ni-NTA superflow resins (Qiagen, Hilden, Germany) (26). The purity of the proteins was estimated by fractionation on SDS-PAGE.

Printing and Processing of Reverse Phase Protein Microarrays. Samples from patient biopsies were diluted in PBS to a final protein concentration of 1 μg/μl before

printing. Aliquots of the recombinant proteins were serially diluted (0 to 10 ng/μl) in PBS containing 0.05% of Tween 20 (PBS-T). Serially diluted protein extracts (0 to 1 μg/μl) derived from HCT-116 colocarcinoma cells were also prepared to asses printing quality and the linear response of protein recognition by the antibodies used. A solution of BSA (1 μg/μl) was also prepared for printing as internal negative control. Approximately, 1 nl volume of each sample was spotted in duplicate onto nitrocellulose-coated glass slides (FAST Slides, Scheleicher & Schuell BioScience, Inc.) using a BioOdyssey Calligrapher MiniArrayer printer (Bio-Rad Laboratories, Inc.) equipped with a capillary pin at constant humidity and temperature. After printing, arrays were allowed to dry and further blocked in PBS-T containing 5% skimmed milk. After, the arrays were incubated overnight at 4º C with the indicated concentrations of the following primary monoclonal antibodies (mAbs): clone 11/21-7A8 for anti-β-F1-ATPase (1 μg/ml), clone 17/9-15 G1 for anti-Hsp60 (1 μg/ml), clone 273A-E5 for anti-GAPDH (1 μg/ml) and clone 14/5-21/24 for PK-M2 (1 μg/ml) which have been previously detailed (26). After incubation the arrays were washed with PBS-T and further incubated with a donkey anti-mouse secondary antibody conjugated with alexa-488 (Invitrogen). Microarrays were scanned using a Typhoon 9410 scanner (GE Healthcare, Inc.). The mean fluorescent intensity of the spots was quantified using Image J software (N.I.H., USA) and converted into pg of protein/ng of total protein using the fluorescent intensity units obtained in the respective standard curve of recombinant protein (see Fig. 1).

Statistical analysis. Distribution of molecular markers and other categorical variables were compared by χ^2 and Student's *t*-test. The statistical significance of linear regressions was assessed by Pearson's correlation *t*-test. For the expression profiles of metabolic markers data were reformatted by calculating the log(2) of the expression

level 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. The Fisher's linear discriminant function was used to assign the biopsy of the patients to one of two considered classes: normal or tumor, using the β-F1-ATPase/Hsp60 ratio and the expression levels of GAPDH and PK-M2 as variables. The actual error rate, or misclassification rate, was estimated by the Lachenbruch's holdout procedure as previously described (28). To determine the association between the expression level of the metabolic markers with disease-free survival (DFS) and overall survival (OS) the cutoff point used to define high and low risk groups was the mean value of protein expression in colon tumor samples from relapsed patients. Survival curves were derived from Kaplan-Meier estimates and compared by log-rank test. Cox proportional hazard regression methods were used to study the relationship between survival, clinical-pathological variables and protein expression in univariate models. Hazard ratios are presented with their 95% confidence intervals (95% CI). Statistical test were two-side at the 5% level of significance.

Results

Quantification of markers of the "bioenergetic signature" in colon cancer. Representative protein microarrays, illustrating the expression of the mitochondrial (β-F1-ATPase, Hsp60) and glycolytic (GAPDH, PK-M2) proteins of energetic metabolism in paired normal (N) and tumor (T) biopsies of colon cancer patients are shown in Figure 1. Samples from the forty different patients studied were spotted in duplicate from left to right and from top to bottom. It should be noted that some of the patient biopsies were lost during protein extraction procedures. Increasing amounts of BSA were spotted in the array as a negative control of the assay (Fig. 1). The arrays also contained duplicates of increasing protein amounts of extracts from HCT-116 cells and of the recombinant proteins studied (Fig. 1). Both of these samples showed an increased linear response in fluorescent intensity as the amount of protein increased in the spot (Fig. 1). Consistent with the high affinity and specificity of the mAbs used (26), each array illustrates the specific recognition of the antigen (recombinant protein) in minute amounts of printed protein of HCT-116 extracts and patient biopsies (Fig. 1). No fluorescent signal was observed in BSA containing spots (Fig. 1), indicating the negligible background of the technique by non-specific absorption of the mAbs to the proteins spotted onto the arrays.

The quantification of the expression of each marker in normal (n=36) and tumor (n=38) biopsies (pg/ng of protein) was calculated from the fluorescent intensity signal interpolated in the corresponding standard curve of recombinant protein. A significant increase in the tumor content of the glycolytic GAPDH and PK-M2 and in the mitochondrial Hsp60 was observed when compared to normal tissues (Table 1). No significant changes were observed in β-F1-ATPase content between normal and tumor samples (Table 1). However, the normalized mitochondrial (β-F1/Hsp60 ratio) and

cellular (β-F1/GAPDH ratio) content of β-F1-ATPase were significantly diminished in the carcinomas, illustrating the alteration of the *bioenergetic signature* in colon cancer (Table 1). Table 1 shows the content of each protein in the tumors according to their classification by clinico-pathological variables. The only relevant difference observed was a significant increase in the mitochondrial content of Hsp60 in tumors from older patients (Table 1), what might reflect the alteration of mitochondria during ageing.

The bioenergetic signature of colon cancer. Previous findings have shown that the alteration of the mitochondrial proteome, as revealed by the marked decrease of the β-F1/Hsp60 ratio in the tumors, inversely correlates with the increased expression of several enzymes of glycolysis, indicating that a compromised energy supply by oxidative phosphorylation is compensated by an increase in the rate of glycolysis (6, 12, 29). In support of this idea, the results in Fig. 2 illustrate significant inverse linear correlations between the β-F1/Hsp60 ratio and the tissue content of the glycolytic GAPDH and PK-M2 enzymes. Moreover, a concerted adaptation of the tumor to aerobic glycolysis was supported by the significant direct linear correlation that exists between the tissue content of GAPDH and PK-M2 (Fig. 2).

To asses the biological relevance of the alteration of the bioenergetic signature in colon cancer, the Fisher linear discriminant analysis was applied to the biopsies using as predictor variables the bioenergetic competence of mitochondria (β-F1-ATPase/Hsp60 ratio) and the tissue content of the two enzymes of glycolysis (GAPDH and PK-M2). Using cross-validation, it was observed that the overall correct classification of the 74 biopsies studied was 85.1%, with a specificity of 91.7% and a sensitivity of 78.9%. In other words, the alteration of the mitochondrial proteome and concurrent induction of markers of glycolysis could also be considered a generalized protein signature of energetic metabolism in colon cancer.

Association of proteomic variables with patient survival. Kaplan-Meier survival analysis of the tumor content of the proteins of energetic metabolism with overall (OS) and disease free (DFS) survival of the patients revealed that a tumor content of PK-M2 above 12.3 pg/ng protein significantly correlated with greater chances of disease recurrence (Fig. 3A). Univariate Cox regression analysis further confirmed the significant association of the tumor content of PK-M2 with DFS [Hazard Ratio=1.14 (95% CI 1-1.3) (p=0.05)]. No significant correlations were obtained between patients' survival and the tumor content of the other proteins of energetic metabolism studied (data not shown). However, and consistent with previous qualitative findings obtained in large cohorts of colon (12, 13) and breast (29) cancer patients it was found that a low β-F1/Hsp60 ratio, which is indicative of a diminished bioenergetic competence of the organelle, also correlated with DFS (Fig. 3B). It was found that a β-F1/Hsp60 ratio below 1.2 correlated with a shorter-time period for recurrence of the disease although this association did not reach the level of statistical significance (Fig. 3B), most likely because the limited size of the cohort of patients studied. In contrast to this finding the β-F1/GAPDH ratio that provides a normalized expression of the overall bioenergetic activity of mitochondria in the cell significantly correlated with both the OS (Fig. 3C) and DFS (Fig. 3D) of the patients. Indeed, patients with β-F1/GAPDH ratios below 0.35 revealed both a worst prognosis (Fig. 3C) and earlier recurrence of the disease (Fig. 3D). Univariate Cox regression analysis also suggested the association of the tumor β-F1/GAPDH ratio with OS [Hazard Ratio=0.01 (95% CI 0-1.5) (p=0.07)]. In other words, more than 70% of patients with a β-F1/GAPDH ratio higher than 0.35 were alive after 5 years study while less than 18% of patients with a β-F1/GAPDH ratio below 0.35 survive this period of time (Fig. 3C).

The "bad" and "good" signatures of colorectal cancer. Unsupervised hierarchical clustering of the 38 tumor biopsies using the three markers (PK-M2 and β-F1/Hsp60 and β-F1/GAPDH ratios) that correlated with survival (Fig. 3) is shown in Figure 4A. The analysis revealed the distribution of the tumors into two groups, C1 and C2 clusters, according to the degree of dissimilarity of the markers studied. Cluster C1 grouped tumors with higher bioenergetic signatures and a lower protein load of PK-M2 when compared to cluster C2 (Fig. 4A and Table II). Consistent with previous results (Fig. 3) patients bearing tumors with the C1 signature had less recurrence of the disease (Fig. 4B) and better overall prognosis (Fig. 4C) when compared to those in C2 as assessed by Kaplan-Maier analysis. In fact, cluster C2 mainly gathered biopsies from patients that relapsed or died along the time of study (Fig. 4A). These findings indicate that a compromised mitochondrial activity and enforced glycolysis afford a phenotypic advantage for colon cancer progression. Consistent with the results in Table 1, no significant differences in the distribution of patients in C1 and C2 cluster in relation with sex, age, tumor histology, tumor grade, nodal affectation and clinical stage were observed (Table 2), indicating the lack of association of markers of energetic metabolism with clinical variables. However, and consistent with previous findings (Fig. 3) it should be noted that univariate Cox regression analysis indicated the significant association of the C2 signature of the tumor with both a worst overall [Hazard Ratio=2.75 (95% CI 0.99-7.62) (p=0.05)] and disease-free [Hazard Ratio=3.48 (95% CI 1.03-11.73) (p=0.04)] survival of the patients.

Discussion

Over the last decade, progress has been made in genomic profiling of cancer patients in an attempt to individualize chemotherapy. However, it is nowadays accepted that successful individualize chemotherapy requires the incorporation of new proteomic techniques of profiling that could actually inform of the functional activity of potential targeted pathways (25). In this regard, RPMA offers a high-throughput technology for quantitative determination of proteins in biopsy material allowing the identification of diagnostic and therapeutic markers, the establishment of correlations with patients' outcome, predicting treatment response and eventually designing a rationale therapy (25, 30). In this regard, the energetic metabolism of tumors has become a central issue of investigation and investment in cancer biology (8-11), mostly because it provides a phenotypic trait of proliferating cells that results in a common *bioenergetic signature* for tumors arising in different tissues (26). In other words, markers of energetic metabolism provide generic targets for cancer treatment. In this study we have applied RPMA technology to quantify the expression of four proteins that inform of the activity of energetic metabolism (6, 15) in normal and tumor samples of CRC patients as an additional effort to stimulate the translation of the *bioenergetic signature* to bed-side application. This step forward is possible because we have previously developed high affinity and specific monoclonal antibodies against these proteins (26) which are the rate-limiting tools required for the successful application of RPMA technology (25).

One of the most prominent alterations found in human tumors is the increased expression of enzymes of glycolysis (12, 28, 29, 31). Consistent with previous qualitative findings in CRC (12), we now document the actual protein quantity and tumor increase of GAPDH and PK-M2 in CRC when compared to normal tissue. The findings are in line with previous data in lung, breast and esophageal carcinomas (26).

PK-M2 expression is restricted to embryonic, cancer and proliferating cells (32, 33) and provides a biomarker for gastric, renal, lung, melanoma and colorectal malignancies (34-37). In agreement with these observations, we found that the quantity of PK-M2 in CRC significantly associated with the time for recurrence of the disease. Regarding changes in the tumor quantity of the two mitochondrial proteins we found a two-fold increase in content of Hsp60 and no relevant changes in β-F1-ATPase when compared to normal samples (Table 1). These findings contrast with our previous qualitative estimations of β-F1-ATPase and Hsp60 expression in CRC determined by immunohistochemistry in tissue microarrays (12). In that study, we found that tumor β-F1-ATPase expression was reduced in the absence of tumor changes in Hsp60 expression (12). It is unlikely that these differences result from changes in the methodology used to determine the expression of the proteins. Rather, we suggest that they arise from large differences in the size and degree of heterogeneity of the two cohorts of CRC patients analyzed. In fact, in the first study (12) we analyzed a large and homogeneous cohort of Stage II CRC patients whereas this study gathered a heterogeneous Stage I to IV cohort of CRC patients (Table 1). It is likely that sample variability, progression of the disease and numbers of studied patients contribute to mitigate (β-F1-ATPase) or enlarge (Hsp60) the content of these proteins. However, both studies perfectly coincide in emphasizing that the normalized expression of β-F1-ATPase or relative tissue content of the protein as assessed by the *bioenergetic signature* (β-F1-ATPase/GAPDH ratio) provide relevant information for patients' overall survival and time for disease recurrence (12).

A low *bioenergetic signature* in breast (29, 38) and lung (15, 28) carcinomas also indicated a worse prognosis for the patients. Therefore, we suggest that the down-regulation of the *bioenergetic signature* of CRC provides a validated biomarker of

disease progression because it has already been demonstrated in three different cohorts of 104 Korean (12), 153 Taiwanese (13) and 40 Spanish CRC patients (this study). Moreover, the genetic alterations of oxidative phosphorylation genes in an additional cohort of 180 US CRC patients further support the clinical utility of the bioenergetic signature in colon cancer prognosis (14).

Down-regulation of oxidative phosphorylation and concurrent activation of aerobic glycolysis is a hallmark feature of proliferating cells and of many different human carcinomas (8). Several mechanisms have been described to promote the so-called Warburg phenotype of the tumors (for review see (8)). Specifically, and at the H^+-ATP synthase level, it has been described: (i) the down-regulation of the cellular abundance of the mRNAs that encode rate-limiting subunits of the complex by either promoter hypermethylation of the ATP5B gene (17) or by genetic deletion of ATP5A1 (14); (ii) the masking of the translation of β-F1-ATPase mRNA (39, 40) through the binding of repressor proteins that target essential *cis*-acting elements of the mRNA impeding ribosome recruitment and translation (41) and (iii) by over-expression in cancer cells and tumors of IF1, an inhibitor of the mitochondrial H^+-ATP synthase (42). Since the bioenergetic phenotype of cells is tissue-specifically expressed and regulated by the microenvironment (6, 43, 44) we suggest that the study of interactors (38) and regulators (41, 42) of the H^+-ATP synthase in different cohorts of cancer patients using RPMA technology will contribute to the identification of additional tissue-specific markers of cancer prognosis and therapy.

The altered energetic metabolism of cancer cells has been proposed as a potential target for cancer treatment (8-11). In fact, the expression level of β-F1-ATPase is a known therapeutic response marker in different cancer cell lines, both for single and combined chemotherapy (6, 13, 16-21). In the specific case of colon cancer cells, the

bioenergetic signature inversely correlates with the potential to execute necrosis in response to treatments with glycolytic inhibitors (18, 19, 21) whereas it directly correlates with the apoptotic response to 5-FU treatment (6, 20, 21). Moreover, it has been described that the 5-year disease-free survival rate of CRC patients receiving 5-FU based chemotherapy is significantly lower in patients with low tumor expression of β-F1-ATPase than in those with high expression (13). Overall, we sustain that these findings support the implementation of the bioenergetic signature in the clinics as a gauge for predicting tumor recurrence in response to different therapeutic strategies. Some advantages of the proteins of energetic metabolism as predictive biomarkers over those of the human "kinome", the complement of kinases encoded in the genome, are that the former group is less promiscuous and less likely to change after procurement of the surgical specimen than kinases (25). Therefore, we encourage the application of RPMA technology in prospective CRC studies using markers of energetic metabolism because they will likely contribute to predict a successful individualized therapy in colon cancer.

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Author contributions:

MA, CNA, MC and MV carried out experiments. JC and CN contributed in the diagnosis, review and clinical classification of the patients included in the study. MA, JC, JRB, MSA and JMC contributed in the analysis of the data. MA and JMC designed experiments and wrote the manuscript. All authors have read and approved the final version of the manuscript.

Competing interests:

JMC as inventor and the Universidad Autónoma de Madrid hold the following patents on "the bioenergetic signature of cancer", which has been licensed to Fina Biotech, S.L. (Spain): US 10/514.771, Japanese 4235610, Canadian 2,487,176 and EU 03 727 509.6. MA, JC, CNA, MC, MV, JRB, MSA and CN declare no competing interests.

Figure Legends.

Figure 1. RPMA of the bioenergetic signature in CRC patients. Representative protein microarrays of β-F1-ATPase (**A**), Hsp60 (**B**), GAPDH (**C**) and PK-M2 (**D**) used for quantification of the bioenergetic signature are shown. Paired normal (N, black boxed) and tumor (T, red boxed) biopsies of each cancer patient (P1 to P40) were spotted in duplicate. Increasing amounts of BSA (0-1 μg/ml), extracts from HCT 116 cells (0-1 μg/ml) and of each of the recombinant proteins (r-) (0-1 ng/ml) were also spotted in the arrays. Highly significant linear correlations exist between the fluorescence intensity (F.I.) (arbitrary units) of the spots and the amount of recombinant protein or native protein in HCT-116 lysates (panels to the right). Protein concentrations in the biopsies were calculated according to the F.I. obtained in standard r-protein curves.

Figure 2. Linear correlations between markers of energetic metabolism. The top two plots illustrate the significant inverse correlations that exist between the bioenergetic competence of mitochondria, estimated by the β-F1-ATPase/Hsp60 ratio, and the protein content of GAPDH and PK-M2 in tissue biopsies. The lower plot shows the direct linear correlation between both markers of glycolysis. Pearson's significance is indicated in each plot.

Figure 3. The bioenergetic signature and patient survival. Kaplan-Meier survival analysis shows the association of the content (PK-M2), the bioenergetic competence of mitochondria (β-F1-ATPase/Hsp60 ratio) and the bioenergetic signature (β-F1-ATPase/GAPDH ratio) of CRC with disease-free-survival (DFS) and overall survival (OS) of the patients. Patients (numbers on top of each trace) were stratified into two groups depending on high (red) or low (green) values of the variable. Log-rank significance is indicated in each plot.

Figure 4. Classification of CRC by their bioenergetic signature. A, Hierarchical clustering of 38 CRC by the mitochondrial competence (β-F1/Hsp60 ratio), bioenergetic signature (β-F1/GAPDH ratio) and content of PK-M2. Rows, samples; columns, markers. Color scale: red, high; black, normal; green, low. The dendrogram provided two major clusters, C1 (blue) and C2 (green). * and $^{+}$, identify patients who developed metastasis or died from the disease, respectively. **B** and **C**, Kaplan-Meier survival analysis show that patients belonging to C1 (blue) had a significant advantage in both DFS (**B**) and OS (**C**) when compared with patients in C2 (green). The number of patients in each cluster is indicated on top of each trace. Log-rank significance is indicated in the plots.

Table I. Clinicopathological characteristics and expression of metabolic markers in human colorectal samples. Tumor histological subtypes: C and R, for colon and rectal carcinomas, respectively. The expression of the markers is shown in pg/ng protein in the biopsy and are mean ± SEM. Bold typed text indicates a significance of $p < 0.05$ by Student's t-test when compared with normal and age <70, respectively.

Characteristics	No.	β-F1-ATPase	Hsp60	β–F1/Hsp60	GAPDH	β–F1/GAPDH	PK-M2
Normal	36	2.15±0.12	0.89±0.05	2.46±0.08	3.91±0.37	0.64±0.04	4.49±0.27
Tumor	38	2.03±0.13	**1.84±0.23**	**1.45±0.10**	**5.81±0.45**	**0.40±0.02**	**10.50±0.79**
Sex							
F	13	1.92±0.21	2.12±0.51	1.27±0.20	5.82±0.76	0.35±0.03	10.64±1.29
M	25	2.09±0.16	1.70±0.23	1.54±0.12	5.81±0.57	0.42±0.03	10.43±1.01
Age							
<70	15	1.82±0.17	1.32±0.18	1.57±0.15	5.25±0.68	0.39±0.03	9.45±1.30
>70	23	2.17±0.18	**2.19±0.35**	1.37±0.14	6.18±0.59	0.40±0.03	11.19±0.98
Histology							
C	27	1.98±0.13	1.66±0.21	1.52±0.11	5.46±0.51	0.42±0.03	9.90±0.75
R	11	2.17±0.31	2.30±0.60	1.27±0.22	6.69±0.90	0.33±0.02	11.98±2.01
Nodes							
No	17	2.03±0.18	1.93±0.42	1.48±0.16	5.44±0.62	0.41±0.03	9.86±1.03
Yes	21	2.04±0.18	1.18±0.26	1.42±0.14	6.12±0.65	0.38±0.04	11.02±1.17
Stage							
I	4	2.29±0.39	1.38±0.45	1.94±0.38	5.45±2.23	0.45±0.04	8.08±2.30
II	10	1.91±0.25	1.88±0.64	1.51±0.19	4.68±0.74	0.44±0.03	9.85±1.36
III	16	2.23±0.19	2.17±0.33	1.35±0.16	6.72±0.68	0.38±0.04	11.44±1.13
IV	8	1.65±0.31	1.39±0.32	1.33±0.22	5.59±1.18	0.34±0.07	10.65±2.29
Grade							
1	6	2.17±0.29	1.42±0.32	1.74±0.25	4.87±0.93	0.51±0.08	8.01±1.22
2	27	2.08±0.15	1.97±0.30	1.42±0.12	6.19±0.55	0.38±0.02	11.09±1.00
3	5	1.60±0.39	1.68±0.63	1.24±0.31	4.90±1.22	0.33±0.04	10.33±2.02

Table II. Clinicopathological characteristics and expression of metabolic markers in C1 and C2 clusters. Values in the categorical variables are expressed as percentage of the tumors in that cluster. Categorical variables were compared by $\chi 2$. The expression of the markers is shown in pg/ng protein in the biopsy and are mean ± SEM. Bold typed text indicates a significance of $p < 0.05$ when compared with C1 by Student's t-test.

Characteristics	C1		C2		χ^2	*P-value*
	n	Value	*n*	Value		
Categorical						
Sex					0.159	0.690
F	8	32	5	38.5		
M	17	68	8	61.5		
Age						
<70	11	44	4	30.8	0.627	0.429
>70	14	66	9	69.2		
Histology						
C	19	76	8	61.5	0.870	0.351
R	6	24	5	38.5		
Nodes						
No	12	48	5	38.5	0.315	0.575
Yes	13	52	8	61.5		
Stage						
I	4	16	0	0	4.510	0.211
II	8	32	2	15.4		
III	9	36	7	53.8		
IV	4	16	4	30.8		
Grade						
1	6	24	0	0	3.706	0.157
2	16	64	11	84.6		
3	3	12	2	15.4		
Numerical						
β-F1-ATPase	25	1.86±0.16	13	2.37±0.19		0.060
Hsp60	25	1.28±0.18	13	2.93±0.46		**0.004**
β-F1/Hsp60	25	1.70±0.12	13	0.96±0.10		**0.000**
GAPDH	25	4.50±0.46	13	8.35±0.45		**0.000**
β-F1/GAPDH	25	0.45±0.03	13	0.28±0.02		**0.000**
PK-M2	25	8.39±0.77	13	14.55±1.11		**0.000**