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ORIGINAL ARTICLE

Glycolysis gene expression analysis and selective metabolic advantage in the clinical progression of colorectal cancer

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Production of lactate even in the presence of sufficient levels of oxygen (aerobic glycolysis) seems the prevalent energy metabolism pathway in cancer cells. The analysis of altered expression of effectors causing redirection of glucose metabolism would help to characterize this phenomenon with possible therapeutic implications. We analyzed mRNA expression of the key enzymes involved in aerobic glycolysis in normal mucosa (NM), primary tumor (PT) and liver metastasis (LM) of colorectal cancer (CRC) patients (pts) who underwent primary tumor surgery and liver metastasectomy. Tissues of 48 CRC pts were analyzed by RT-qPCR for mRNA expression of the following genes: hexokinase-1 (*HK-1*) and 2 (*HK-2*), embryonic pyruvate kinase (*PKM-2*), lactate dehydrogenase-A (*LDH-A*), glucose transporter-1 (*GLUT-1*), voltage-dependent anion-selective channel protein-1 (*VDAC-1*). Differences in the expression of the candidate genes between tissues and associations with clinical/pathologic features were studied. *GLUT-1*, *LDH-A*, *HK-1*, *PKM-2* and *VDAC-1* mRNA expression levels were significantly higher in PT/LM tissues compared with NM. There was a trend for higher expression of these genes in LM compared with PT tissues, but differences were statistically significant for *LDH-A* expression only. *RAS* mutation-positive disease was associated with high *GLUT-1* mRNA expression levels only. Right-sided colon tumors showed significantly higher *GLUT-1*, *PKM-2* and *LDH-A* mRNA expression levels. High glycolytic profile was significantly associated with poor prognosis in 20 metastatic, *RAS*-mutated pts treated with first-line chemotherapy plus Bevacizumab. Altered expression of effectors associated with upregulated glucose uptake and aerobic glycolysis occurs in CRC tissues. Additional analyses are warranted for addressing the role of these changes in anti-angiogenic resistance and for developing novel therapeutics.

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INTRODUCTION

Altered cellular energetics metabolism is emerging as one of the most important hallmarks of cancer.[1] Among the several changes of metabolic pathways in tumor cells, a key role is played by enhanced aerobic glycolysis with lactic fermentation, which is also known as the Warburg effect.[2] In fact, normal cells generally transform glucose into carbonic anhydride under aerobic conditions, by means of oxidative phosphorylation. On the contrary, invasive cancer cells mostly produce lactate, even in the presence of sufficient levels of oxygen.[2] Glycolysis, the conversion of glucose to lactate, only produces 2 ATP molecules, compared with 36 for the complete oxidation of glucose to CO_2 and H_2O. Thus, incomplete oxidation of glucose to lactate yields only 5% of the energy available from glucose. This apparently senseless waste of glucose actually constitutes a survival advantage in rapidly proliferating cells, because it makes them insensitive to transient or permanent hypoxic conditions, it contributes to the production of nucleosides and aminoacids, and, thanks to the enhanced glucose uptake occurring in cancer tissues, constitutes a very rapid way to produce energy.[1,2] Furthermore, lactate is not just a waste product of this process; on the contrary, it promotes tumor invasion by favoring cell migration, angiogenesis, immune escape and radioresistance.[3] This redirection of glucose metabolism is promoted by the overexpression of the key effectors of the glycolytic pathway,[4] consisting of specific membrane transporters of glucose (GLUTs), as well as of all the enzymes responsible for the promotion of each single step of the cascade involved in the transformation of glucose into lactic acid (Figure 1). Moreover, the overexpressed enzymes themselves are subject to selection and as a consequence only certain isoforms are majorly represented in tumor cells.[4]

Glucose transporters (GLUTs) constitute a family of proteins that regulate the transport of glucose across the hydrophobic cell membranes.[5] Fourteen isoforms of the GLUT genes have been identified, which show similar structural architecture but different cellular and sub-cellular localization, kinetic properties and affinity for glucose and other hexoses. Among the different GLUTs, GLUT-1 has been found to be frequently upregulated in a wide variety of cancer types, and its levels of expression often correlated with the metastatic potential and worse prognosis.[5] The first step in glycolysis is catalyzed by hexokinase (HK) and consists of the transfer of one phosphate group from ATP to glucose, yielding glucose-6-phosphate. Isoforms 1 and 2 of hexokinase (HK-1 and HK-2) are considered to have relevant roles in the reprogrammed metabolism of tumor cells.[2] First, they trigger a key step in the upregulation of glycolytic rates. Second,

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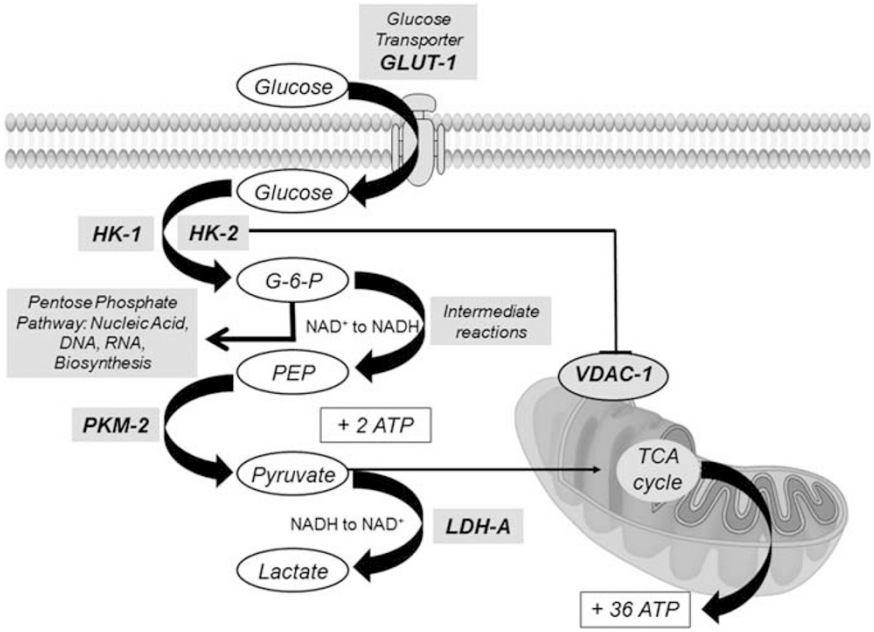

Figure 1. Schematic representation of the anaerobic glycolysis and the oxidative phosphorylation. Tissues first metabolize glucose to pyruvate via glycolysis: hexokinase (HK-1 and HK-2) transfer one phosphate group from ATP to glucose, yielding glucose-6-phosphate (G6P). HK-2 also interacts with the voltage-dependent anion channel (VDAC-1) transporter on the external mitochondrial membrane. G6P may be shunted into the non-oxidative arm of the pentose phosphate pathway (PPP), otherwise it is converted through intermediate reaction of glycolysis to phosphoenolpyruvate (PEP). Pyruvate kinase (PKM-2) catalyzes the transfer of a phosphate group from PEP to ADP, to give pyruvate and ATP. In the presence of oxygen, cells completely oxidize most of that pyruvate in the mitochondria to CO_2 during the process of oxidative phosphorylation in the tricarboxylic acid (TCA) cycle. When oxygen is limiting, cells can redirect the pyruvate generated by glycolysis away from mitochondrial oxidative phosphorylation by generating lactate (anaerobic glycolysis). Lactate dehydrogenase (LDH) catalyzes the reversible conversion of pyruvate to lactate with the simultaneous oxidation of the cofactor NADH to NAD^+. This generation of lactate during anaerobic glycolysis allows glycolysis to continue (by cycling NADH back to NAD^+). Warburg observed that cancer cells tend to convert most glucose to lactate regardless of whether oxygen is present (aerobic glycolysis).

they provide cancer cells of glucose-6-phosphate for the pentose phosphate pathway, which is an important alternative pathway for nucleotide biosynthesis. Finally, the binding of HK-2 to the external mitochondrial membrane in a complex with the voltage-dependent anion channel (VDAC-1) transporter further contributes to the glycolytic shift and the inhibition of apoptosis through block of cytochrome-c release from mitochondria.[6] Pyruvate kinase (PK) catalyzes the final rate-limiting step of glycolysis, which consists in the transfer of a phosphate group from phosphoenolpyruvate to ADP, to give pyruvate and ATP. An upregulation of PKM-2 isoform has been observed in many tumor cells.[7] PKM-2 occurs as a tetramer (highly active) or as a dimer (less active). When cells require energy, tetrameric PKM-2 is prevalent. In contrast, dimeric PKM-2 becomes active when cells enter a proliferative stage.[7] Lactate dehydrogenase (LDH) catalyzes the reversible conversion of pyruvate to lactate with the simultaneous oxidation of the cofactor NADH to NAD^+.[3] The human isoform LDH-A (or LDH5) is mainly expressed in liver and muscle. Several evidences suggest that LDH-A, which is upregulated in invasive glycolytic cancers, has a critical role in cell proliferation, allowing the survival of tumors even in conditions of low oxygen concentration.[3]

There are limited data on the altered expression of glucose metabolic effectors *in vivo*. Regardless of its origin, this information is relevant to the understanding of selective metabolic advantages in invasive cancer tissues.[8] In fact, it would improve our knowledge on a possible mechanism for chemoresistance and secondary resistance to biologic compounds targeting angiogenesis.[9,10] In addition, it may promote the development of new compounds that target cancer metabolism.[11] This background prompted us to the planning of a gene expression study of key enzymes involved in glycolysis. For this purpose, colorectal cancer with liver metastasis was deemed a suitable and relevant model for an *in vivo* analysis.

MATERIALS AND METHODS

We planned a retrospective analysis of patients with metastatic colorectal cancer, who underwent resection of the primary tumor with synchronous or metachronous resection of liver metastasis. This setting would have allowed the availability of paired tissues for comparison of mRNA expression levels between normal mucosa, colorectal cancer and corresponding liver metastasis. The primary end point of the study was the analysis of a possible mRNA overexpression of key enzymes involved in the glycolytic shift in cancer cells (Figure 1). Additional analyses were addressed to the comparison of mRNA expression levels in the primary carcinomas according to clinical/pathologic features. Finally, an hypothesis-generating analysis was planned with an exploratory evaluation of survival outcomes according to mRNA levels in patients with *RAS*-mutated carcinomas treated with chemotherapy plus Bevacizumab. All patient information and pathology material were collected under a protocol approved by the Institutional Review Board.

Patients

Consecutive patients who underwent colorectal cancer surgery with synchronous or metachronous liver metastasectomy at three participating Institutions over a 5-year period (2008–2013) were considered eligible for study inclusion. Full clinical and follow-up information, analysis of *RAS* mutational status, together with the availability of archival paraffin-embedded colonic/tumor/metastasis tissues were required. Patients were excluded if they had a diagnosis of diabetes mellitus (or assumption of active anti-diabetic drugs). To ensure for reproducible and affordable expression assays, patients were also excluded if they had received

Table 1. Characteristics of the 48 patients

	No of patients
Age (years)	
>70 years	20 (40%)
$\leqslant 70$ years	28 (60%)
Sex	
Male	31 (64%)
Female	17 (36%)
Primary tumor site	
Right colon	12 (25%)
Left colon	36 (75%)
Loco-regional lymph nodes	
Negative (pN0)	14 (29%)
Positive (pN1, pN2, pN3)	34 (71%)
Timing of liver metastasis	
Synchronous	32 (66%)
Metachronous	16 (34%)
Tumor grading	
Grade 1–2	38 (79%)
Grade 3	10 (21%)
KRAS/NRAS mutation	
Positive	23 (48%)
Wild type	25 (52%)
First-line chemotherapy	
Fluoropyrimidine/Oxaliplatin plus Bevacizumab	12 (25%)
Fluoropyrimidine/CPT-11 plus Bevacizumab	10 (21%)
Fluoropyrimidine/CPT-11 plus Cetuximab	6 (12%)
Fluoropyrimidine/Oxaliplatin	3 (6%)
Fluoropyrimidine/CPT-11	3 (6%)
Fluoropyrimidine monotherapy	1 (2%)

systemic anti-cancer therapy for metastatic disease before liver metastasectomy

Samples

Four to six 10-µm sections from formalin-fixed, paraffin-embedded specimens were obtained from the primary tumor and the liver metastasectomy. Before cutting sections for mRNA isolation, an additional slide was prepared for hematoxylin and eosin staining and the pathologists identified representative areas with almost complete representation of normal colonic mucosa and tumor infiltration. Sections were sent to the Laboratory of Molecular Biology, Department of Molecular Sciences, University of Urbino for centralized analyses of mRNA levels. All assays were performed by investigators who were blinded to clinical data of the sample cohort. For each enrolled patient, mRNA was extracted from the normal colonic mucosa, from the primary colorectal carcinoma and the corresponding liver metastasis. Real-time-quantitative PCR (RT-PCR) was adopted for mRNA expression analysis of the following genes: Hexokinase-1 (*HK-1*), Hexokinase-2 (*HK-2*), Embryonic Pyruvate Kinase (*PKM-2*), Lactate Dehydrogenase-A (*LDH-A*), Glucose Transporter-1 (*GLUT-1*), Voltage-Dependent Anion-Selective Channel Protein-1 (*VDAC-1*).

Total RNA extraction and cDNA synthesis

All sections were micro-dissected and placed in a 1.5 ml reaction tube containing 1 ml xylene. The deparaffinization procedure was carried out in the tube. Total RNA from formalin-fixed, paraffin-embedded material was extracted using the RNeasy FFPE kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration and purity of the sample (three samples from each patient: normal mucosa, primary and corresponding metastatic tumor) was measured using the NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Rockland, DE, USA). SuperScript$^{(R)}$ VILO^{TM} cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA) was used to generate first-strand cDNAs from 2 µg of total RNA according to the manufacturer's instructions. The real-time products were diluted to obtain a final concentration of 15 ng/µl of reverse-transcribed mRNA.

Quantitative real-time polymerase chain reaction

Quantitative real-time PCR (RT-qPCR) was performed to analyze the mRNA expression levels of the six candidate genes and two reference genes, *B2M* (b2-Microglobulin) and *GUSB* (b-Glucuronidase). To ensure the reproducibility of results, mRNA relative expression analysis of each candidate gene was performed adopting a primary reference gene (*B2M*) and it was repeated adopting a second reference gene (*GUS*). RT-qPCR was carried out using TaqMan Gene Expression Assay and TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 7500 Real Time PCR System. The 20 µl of PCR mixture included 4 µl diluted RT product, 10 µl 2X TaqMan Gene Expression Master Mix, 1 µl of 20X TaqMan Gene Expression Assay and 5 µl of distilled water. The reaction mixture was incubated at 50 °C for 2 min (for UNG activity) followed by 95 °C for 15 min and by 40 amplification cycles at 95 °C for 15 s (denaturation), 60 °C for 1 min (annealing/extension). All reactions were performed in quadruplicate and each PCR run included a no template control.

Relative mRNA quantification

The relative mRNA expression of each candidate gene was expressed as the $\Delta Ct = Ct_{(target\ gene)} - Ct_{(reference\ gene)}$. The reference gene selection was based on widely published housekeeping genes used in colorectal cancer expression profile studies.[12] The Ct is defined as the number of cycles needed for the fluorescence to reach a specific threshold level of detection selected (0.02) and it is inversely correlated with the amount of nucleic acid template present in the sample.[13] Therefore, a higher mRNA expression level corresponds to a smaller ΔCt value and a difference in 1 unit between ΔCt values approximately corresponds to doubling of mRNA expression.[13] To this regard, in a further step, a second relative parameter was added as produced by the $2^{-\Delta\Delta Ct}$ method.[14] It expresses the n-fold differential expression (fold change) of the candidate gene between the tumor sample and the normal counterpart. Fold change analysis further defines the magnitude of the differential expression possibly representing biological relevance.

Statistical analysis

The mRNA expression levels reported as ΔCt values with mean and s.d. were compared between each analyzed group adopting the *t*-test and the Wilcoxon test. All reported *P*-values were two-sided, and confidence intervals were at the 95% level. A $P\text{-value} < 0.05$ was considered statistically significant. Significant associations were required to be detectable with both reference genes. In addition, to address the issue of conducting multiple tests (six gene expressions), we applied the false discovery rate control method of Benjamini and Hochberg. The Pearson correlation coefficients were used to assess the correlation of the mRNA expression levels between different genes. Intersection of fold change $\geqslant 2$ with significant *P* values at the ΔCt level analysis was adopted to further characterize overexpressed genes at a putative biological relevant threshold in the 48 tumors.[15]

In the exploratory overall survival (OS) analysis, OS was calculated starting from the date of the first cycle of first-line chemotherapy to date of death or last follow-up. Univariate Cox proportional hazards regression was used to evaluate the association between OS and $2^{-\Delta\Delta Ct}$ values of differentially expressed genes between normal and primary tumor tissue. A multivariable Cox proportional hazards model was then used to develop a predictive model of OS based on the gene expression values.[16] For each patient, the $\sum_{pj} = 1 x_j \beta_j$ Linear Predictor was estimated and High/Low Glycolytic Risk Score (GRS) was generated according to the median of the Linear Predictor values as the threshold value.[16,17] The Kaplan–Meier method was used to estimate survival curves and the log-rank test was used to compare OS between high and low risk groups. Analyses were performed with SAS 9.2 (SAS Institute, Cary, NC, USA).

RESULTS

Fifty patients were included in the study. However, two patients were excluded because of technically unassessable archival

Table 2. Results of the mRNA expression analysis

Variable	*Mean*[a]	*s.d.*[a]	*Median (q1-q3)*[a]	*Comparison*	*DF*	T-*test*	*Wilcoxon*
						P-*value*	P-*value*
B2M reference gene							
HK-1 normal (N)	6.8	1.0	6.9 (6.3–7.5)	T vs N	47	0.0474	0.0349
HK-1 tumor (T)	6.7	0.8	6.6 (6.2–7.4)	T vs M	47	0.4711	0.5851
HK-1 metastasis (M)	6.4	1.0	6.5 (6.0–7.2)	M vs N	47	0.0429	0.0420
HK-2 normal (N)	11	1.7	10 (9.2–12)	T vs N	46	0.7350	0.5591
HK-2 tumor (T)	10	1.5	10 (9.3–12)	T vs M	46	0.8259	0.9400
HK-2 metastasis (M)	10	1.5	10 (9.3–11)	M vs N	46	0.6875	0.9419
LDH-A normal (N)	6.7	1.1	6.7 (6.0–7.2)	T vs N	47	< 0.0001	< 0.0001
LDH-A tumor (T)	5.1	1.1	5.2 (4.3–5.7)	T vs M	47	0.0031	0.0030
LDH-A metastasis (M)	4.5	1.4	4.4 (3.5–5.4)	M vs N	47	< 0.0001	< 0.0001
PKM-2 normal (N)	9.1	1.0	9.2 (8.1–9.8)	T vs N	46	< 0.0001	< 0.0001
PKM-2 tumor (T)	7.4	1.4	7.2 (6.4–8.2)	T vs M	46	0.5349	0.3741
PKM-2 metastasis (M)	7.3	1.7	7.2 (5.7–8.3)	M vs N	47	< 0.0001	< 0.0001
GLUT-1 normal (N)	8.3	1.1	8.2 (7.8–8.8)	T vs N	47	< 0.0001	< 0.0001
GLUT-1 tumor (T)	5.8	1.6	6.0 (4.7–7.0)	T vs M	47	0.6328	0.3150
GLUT-1 metastasis (M)	5.7	1.9	5.7 (4.2–6.7)	M vs N	47	< 0.0001	< 0.0001
VDAC-1 normal (N)	8.0	1.3	7.8 (7.3–8.8)	T vs N	47	< 0.0001	< 0.0001
VDAC-1 tumor (T)	6.2	1.1	6.0 (5.4–6.9)	T vs M	47	0.3973	0.4660
VDAC-1 metastasis (M)	6.4	1.4	6.4 (5.3–7.2)	M vs N	47	< 0.0001	< 0.0001
GUS reference gene							
HK-1 normal (N)	2.6	1.0	2.8 (1.6–3.4)	T vs N	45	0.0491	0.0411
HK-1 tumor (T)	2.2	1.0	2.3 (1.5–3.0)	T vs M	45	0.4850	0.5751
HK-1 metastasis (M)	2.0	0.9	2.1 (1.4–2.8)	M vs N	45	0.0070	0.0001
HK-2 normal (N)	6.3	2.0	6.2 (5.0–7.7)	M vs N	45	0.0825	0.0798
HK-2 tumor (T)	6.3	2.0	6.4 (5.0–7.9)	T vs M	41	0.8167	0.8012
HK-2 metastasis (M)	5.7	2.3	5.9 (4.8–7.3)	T vs N	41	0.2004	0.0723
LDH-A normal (N)	1.8	1.7	1.9 (0.5–3.0)	T vs N	45	< 0.0001	< 0.0001
LDH-A tumor (T)	0.8	1.5	0.8 (−0.5 – 1.6)	T vs M	45	0.0542	0.0449
LDH-A metastasis (M)	0.4	1.7	0.1 (−0.7 – 1.8)	M vs N	45	< 0.0001	< 0.0001
PKM-2 normal (N)	4.2	1.5	4.4 (3.4–5.3)	T vs N	44	< 0.0001	< 0.0001
PKM-2 tumor (T)	3.0	1.7	2.8 (1.8–3.7)	T vs M	44	0.5781	0.5821
PKM-2 metastasis (M)	3.1	2.0	3.0 (1.5–4.7)	M vs N	45	< 0.0001	< 0.0001
GLUT-1 normal (N)	3.5	1.1	3.6 (2.8–4.2)	T vs N	45	< .0001	< 0.0001
GLUT-1 tumor (T)	1.5	1.7	1.9 (0.5–2.8)	T vs M	45	0.9318	0.4603
GLUT-1 metastasis (M)	1.5	1.8	1.1 (0.2–2.8)	M vs N	45	< 0.0001	< 0.0001
VDAC-1 normal (N)	3.1	1.8	3.3 (2.1–4.3)	T vs N	45	< 0.0001	< 0.0001
VDAC-1 tumor (T)	1.9	1.6	1.6 (0.7–2.9)	T vs M	45	0.0434	0.0437
VDAC-1 metastasis (M)	2.2	1.7	2.1 (1.1–3.6)	M vs N	45	0.0004	0.0010

Abbreviation: DF, degrees of freedom. [a]Data are presented as ΔCt values: the smaller the ΔCt value, the higher is the expression.

samples. The characteristics of the remaining 48 patients are listed in Table 1. The *RAS* mutation status was positive in 23 patients, with 20 (41.6%) and 3 (6.2%) carcinomas harboring *KRAS* and *NRAS* mutations, respectively. First-line chemotherapy for metastatic disease was administered to 35 patients (73%) with progression after metastasectomy. In 22 patients, it was a combination chemotherapy regimen plus the anti-angiogenic Bevacizumab.

Expression analyses

As shown in Table 2, statistically significant differences in mRNA expression levels were detected between the primary tumor tissues and normal mucosa for *GLUT-1*, *PKM-2*, *LDH-A*, *VDAC-1* and *HK-1*. The mRNA expression of these candidate genes was also significantly higher in metastatic tissues compared with normal mucosa. These statistically significant differences were confirmed after the false discovery rate test control. Globally, metastatic tissues showed a trend for higher mRNA expression of the candidate genes in comparison with primary colorectal tumor tissues. However, a statistically significant difference was detected for *LDH-A* expression only (Table 2).

In Figure 2 are shown the statistically significant differences that were detected in the analysis of mRNA expression levels according to clinical/pathologic features (Table 1). Among the six candidate genes, *GLUT-1* mRNA analysis showed higher expression in *RAS*-mutated than in *RAS* wild-type carcinomas. Metastatic regional lymph node involvement was significantly associated with high *GLUT-1* and *HK-1* mRNA expression levels. Right-sided colon carcinomas showed significantly higher *GLUT-1*, *PKM-2* and *LDH-A* mRNA expression levels.

The ranking of fold change analysis is reported in Figure 3. *GLUT-1* showed the highest increase in mRNA amount in tumor tissues (12.7 mean fold change) and metastatic tissues (17.9 mean fold change). *LDH-A* mean fold change was 4.3 in the primary colorectal cancer tissue and it was more than twice in the liver metastasis tissue (9.4 mean fold change). *HK-1* and *HK-2* showed the lowest mean fold changes. Among the five candidate genes with significant values at the ΔCt analysis level (*GLUT-1*, *LDH-A*, *PKM-2*, *VDAC-1*, *HK-1*), fold change $\geqslant 2$ was detected in 39 cases for *GLUT-1*, in 36 cases for *LDH-A*, in 34 cases for *PKM-2*, in 37 cases for *VDAC-1* and in 20 cases for *HK-1* (Figure 3).

Survival analysis

As previously stated, we performed a hypothesis-generating analysis for association of mRNA expression levels of the candidate genes and survival of the 20 patients with *RAS*-mutated

tumors who were treated with first-line combination chemotherapy plus Bevacizumab. Significant associations with OS after univariate and multivariate procedures (data not shown) indicated *HK-1*, *LDH-A*, *GLUT-1* and *PKM-2* to be included in the GRS model.

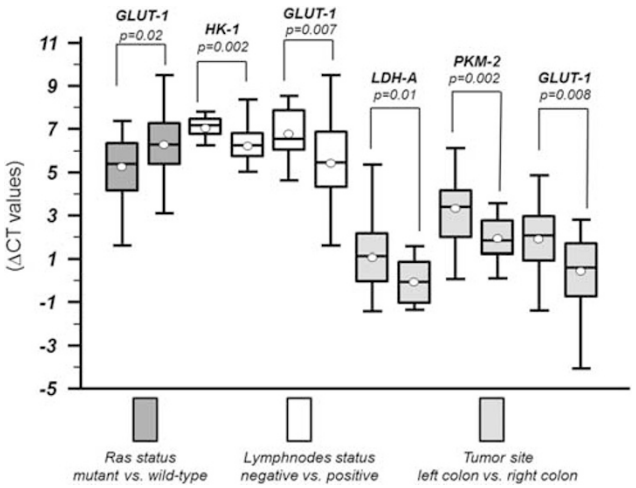

Figure 2. Significant associations detected when mRNA expression levels of the candidate genes were related to clinical and pathological features of the 48 patients. Data are presented as ΔCt values: the smaller the ΔCt value the higher is the expression. Dark gray boxes denote analyses in *RAS*-mutated tumors (left box) and *RAS* wild-type tumors (right box). White boxes indicate analyses in Lymph node-negative tumors (left boxes) and Lymph node-positive tumors (right boxes). Gray boxes indicate analyses in left-sided carcinomas (left boxes) and right-sided carcinomas (right boxes).

However, *PKM-2* and *GLUT-1* levels showed high correlation, with *GLUT-1* possessing the strongest association with OS. Therefore, the multivariate model for producing the GRS included *HK-1*, *LDH-A* and *GLUT-1*. According to the GRS analysis, GRS-high patients were defined as those having risk score greater than the group median. As shown in Figure 4, there was a significantly reduced OS for GRS-high patients in the tumor analysis cohort. Median OS was 24 months in patients with GRS-low and 17.6 months in patients with GRS-high.

DISCUSSION

The metabolic dependencies of cancer cells can be exploited for cancer treatment and downregulation or inhibition of glycolysis has demonstrated therapeutic activity in experimental models.[11] A number of molecules have been found to be blockers of the GLUT and the glycolytic enzymes HK-1, HK-2, PKM-2, LDH-A and VDAC-1, all of which regulate irreversible and rate-limiting steps in glycolysis.[11] Forthcoming clinical trials adopting these new, and hopefully effective, anti-cancer therapeutics would benefit from improved knowledge on the 'metabolic' target expression and its driver role in the tumor phenotype. Indeed, the results of our study support the existence of an upregulated 'glycolytic' profile in colorectal carcinomas. In addition, the acquisition of an efficient glycolytic cascade seems to be an early rather than a late event limited to the metastatic dissemination of the disease.

The glucose transporter *GLUT-1* showed the greatest level of overexpression in colorectal cancer tissues, and this finding parallels the established principle that most primary and metastatic human cancers display significantly increased glucose uptake.[1] Our data support a role of the *RAS* oncogene in the upregulation of the first step of glucose metabolism in cancer cells, with an association between *RAS* mutation-positive tumors and *GLUT-1* mRNA overexpression. However, *RAS*-mutated tumors

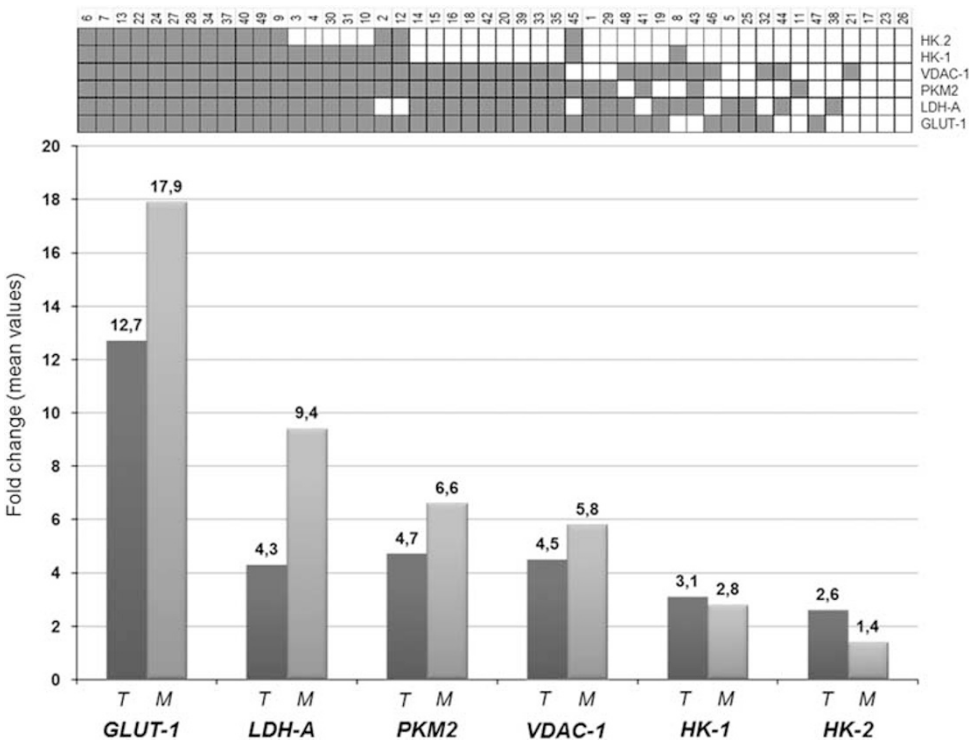

Figure 3. Histograms showing the results and the ranking of the fold change mRNA expression analysis in primary colorectal tumor tissues (T) and metastatic tumor tissues (M) relative to normal mucosa. In the 48 samples, the tumor profiling with genes fold change $\geqslant 2$ (dark gray squares) is shown at the top of the figure.

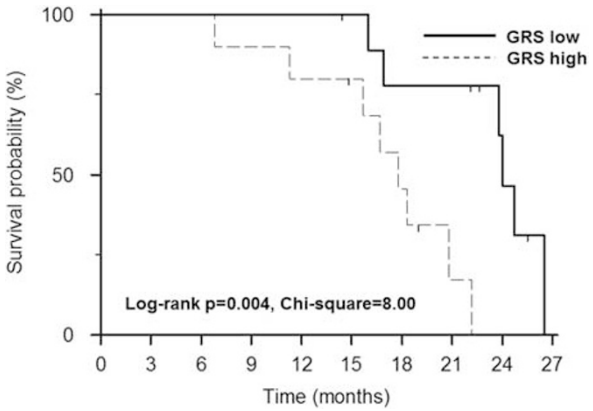

Figure 4. Kaplan–Meier survival curves of the 20 *RAS*-mutated patients treated with first-line chemotherapy plus Bevacizumab and grouped by the Glycolytic Risk Score (GRS).

did not show additional associations with upregulated genes of the 'pro-glycolytic' cascade. Therefore, *RAS* mutations may contribute to the control of the global energy metabolism of cancer cells by promoting glucose uptake, while complex genetic changes (*HIF-1*, *Myc*, *PI3K*, *AKT*, *p53*) may be more closely related to the control of the aerobic glycolysis pathway.[18] We studied the *RAS* mutational status in primary tumors only. However, given the reported high degree of concordance in *RAS* genotyping between primary colorectal carcinomas and matched metastatic sites,[19,20] our strategy should not jeopardize the results of *RAS* correlations.

Among the intermediate key enzymatic activity in the glycolytic cascade, strong (*PKM-2* and *VDAC-1*) and moderate (*HK-1*) upregulation in primary tumors and metastatic tissues was observed. In the last decades, immunohistochemistry studies addressed the question of PKM-2, VDAC-1, HK-1 and HK-2 positivity in several tumor types.[8] However, only in recent years, more refined studies have been planned with mRNA expression and functional analyses in human cancers. The *VDAC-1* expression profile was found to be upregulated in breast, colon, liver, lung, pancreatic and thyroid cancers.[21] *PKM-2* mRNA overexpression showed unfavorable predictive role for platinum-based chemotherapy in non-small cell lung cancer patients.[22] High *HK-2* mRNA expression levels with prognostic impact were reported in ovarian cancer.[23] Our analysis in colorectal carcinomas parallels these findings except for *HK-1* and *HK-2* expression levels that resulted apparently lower than expected. However, to our opinion, these findings do not question the global energy metabolism shift towards a glycolytic profile in colorectal cancer cells. First, it is possible that higher than detected *HK-1* and *HK-2* tumor expression levels are not necessary given the tandem activity of both enzymes in a single enzymatic step. Second, tumor hexokinase isoenzymes were demonstrated to display marked increased affinity to glucose when compared with the corresponding normal prototypes.[24] Third, hexokinases contribute to an important alternative pathway (pentose phosphate pathway) for nucleotide biosynthesis and the colonic mucosa has one of the highest proliferative rates of any adult tissue.[25] Therefore, baseline *HK-1* and *HK-2* upregulation in the normal mucosa may attenuate tumor/mucosa differences in their relative expression profile. Finally, the finding of higher *HK-1* than *HK-2* tumor expression is in line with discoveries from experimental studies suggesting that *HK-1* is the dominant form in tissue with high catabolic (=glycolytic) activity, whereas *HK-2* is better suited for anabolic tasks and *VDAC-1* interaction.[24]

In our study, the remarkable higher *LDH-A* expression levels in primary tumors and liver metastases compared with normal mucosa corroborate the hypothesis of an invasive glycolytic phenotype of colorectal cancers. This last glycolytic step is not only necessary for the final conversion of pyruvate to lactate, but also for the simultaneous oxidation of the cofactor NADH to NAD^+. In turn, adequate NAD^+ levels are necessary for sustaining the intermediate enzymatic glycolytic reactions where NAD^+ is reutilized to form NADH. The finding that *LDH-A* levels were even more upregulated in the metastatic tissues than in the primary colorectal cancers suggests the critical role of this step in maintaining an efficient glycolysis in the tumor progression. High serum LDH levels in metastatic cancer patients have been indicated as a negative prognostic factor[26] and as a negative predictive factor in patients treated with chemotherapy plus Bevacizumab.[27] In our retrospective analysis, we could not collect affordable data on serum LDH pre-treatment levels. However, as previously reported, LDH tumor expression levels may be a more accurate marker of an aggressive colorectal cancer tumor phenotype than LDH serum levels.[28] Notably, we found that *LDH-A* together with *PKM-2* and *GLUT-1* mRNA overexpression characterized the sub-group of patients with right-sided colorectal carcinomas. This sub-group of patients has been recently reported to display an adverse prognosis in patients with metastatic disease treated with chemotherapy with or without Bevacizumab.[29] It could be hypothesized that the acquisition of a marked glycolytic phenotype is among the detrimental effects caused by the accumulation of specific genetic and epigenetic changes that drive the right-sided colorectal tumors.

The possible impact of high glycolytic profile on survival in *RAS*-mutated patients treated with chemotherapy plus Bevacizumab deserves further investigation. This intriguing result is not surprising according to experimental and pre-clinical studies, which showed that glycolysis is an essential metabolic pathway in the hypoxic adaptation for survival and tumor progression.[9] Therefore, it is plausible the hypothesis that tumors develop early and/or late resistance to anti-angiogenic agents when clones are more equipped for prompt and redundant metabolic changes (that is, glycolytic shift) in a therapeutically induced hypoxic environment.[10] In this perspective, the novel compounds with tumor metabolism interference activity, which are currently in pre-clinical or early clinical trials may contribute to an improvement of the overall anti-angiogenic treatment strategy in solid tumors.[11]

Possible limitations of this study are its retrospective small sample size, the lack of protein expression analysis and the lack of correlation with Fluorine-18 fluorodexyglucose positron emission tomography scan. First, it should be considered that we carried out a quite sophisticated study of glycolysis profiling in colorectal cancer tissues including liver metastasis and to the best of our knowledge, this is the first translational analysis with clinical correlates. Indeed, results of this investigation should be looked at with caution and larger confirmatory studies are needed. Second, the correlation between mRNA and protein abundance depends on distinct biological and technical factors and it is difficult to be evaluated on a global level. However, as recently reviewed,[30] mRNA and protein levels may correlate better than previously thought. Third, the Fluorine-18 fluorodexyglucose positron emission tomography scan is not a routine exam in the staging procedures of patients undergoing systemic therapy for metastatic colorectal cancer. A prospective investigation should address this issue, but it would add the obstacle of data analysis and interpretation of results of the PET scan maximum standardized uptake values (SUVmax) related to the glycolytic enzyme expression levels.[31] As far as the survival analysis is concerned, we cannot draw any conclusion on the predictive or the prognostic role of the glycolysis profiling. Considering the possible multiple effects of this feature in maintaining the cancer cell phenotype, both clinical effects may occur. However, the predictive/prognostic analysis would require a properly sized and specific study plan,[32] which was beyond the scope of our

exploratory survival analysis. In fact, we sought to detect an early sign of a possible clinical impact to be explored in future studies.

In conclusion, the results corroborate the endeavors to explore cancer metabolism and identify molecules with metabolism interference properties.[11] We are planning a new study to confirm the clinical impact of the tumor glycolytic profiling in a prospectively enrolled cohort of metastatic, *RAS*-mutated colorectal cancer patients treated with chemotherapy and Bevacizumab.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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