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Transketolase-like protein 1 (TKTL1) is required for rapid cell growth and full viability of human tumor cells

Xiaojun Xu[1], Axel zur Hausen[2], Johannes F. Coy[3] and Martin Löchelt[1]*

[1]*Department of Genome Modifications and Carcinogenesis, Research Program Infection and Cancer, German Cancer Research Centre, Heidelberg, Germany*
[2]*Institute of Pathology, Medical Faculty, University of Freiburg, Freiburg, Germany*
[3]*Tavarlin AG, Darmstadt and Tavargenix GmbH, Würzburg, Germany*

Cancer cells display high rates of aerobic glycolysis, a phenomenon known as the Warburg effect. Lactate and pyruvate, the end products of glycolysis, are overproduced by cancer cells even in the presence of oxygen. The pentose phosphate pathway (PPP) allows glucose conversion to ribose for nucleic acid synthesis, glucose degradation to lactate, and regeneration of redox equivalents. The nonoxidative part of the PPP is controlled by transketolase (TKT) enzymes. One TKT isoform, the transketolase-like protein 1 (TKTL1) is specifically upregulated in different human cancers and its overexpression predicts a poor patient's survival. This finding implicates that an increased TKTL1 expression may activate the PPP leading to enhanced cancer cell growth and survival. To analyze the functional role of TKTL1 in malignant progression, we inhibited TKTL1 by RNAi technologies in human HCT116 colon carcinoma cells. TKTL1 suppression resulted in a significantly slowed cell growth, glucose consumption and lactate production. In TKTL1 knockdown-cells, the intracellular reactive oxygen species levels were not significantly increased, whereas the sensitivity towards oxidative stress-induced apoptosis was clearly enhanced. These data provide new clues on the importance of TKTL1 dys-regulation in tumor cells and indicate that TKTL1 overexpression may be considered not only as a new tumor marker but also as a good target for anticancer therapy.

Key words: TKTL1; aerobic glycolysis; shRNA; reactive oxygen species (ROS)

Cancer is caused by endogenous and exogenous factors leading to the sequential accumulation of genetic alterations, a scenario known as multistep oncogenesis.[1] Organotypically different tumors are often characterized by related or even identical changes in cell physiology and cell metabolism.[2] A characteristic of solid, malignant tumors is the strongly enhanced glycolytic metabolism of carbohydrates even in the presence of oxygen, the so-called aerobic glycolysis or Warburg effect.[3] This feature characterizes cancer metabolism as highly inefficient by breaking down excess amounts of glucose to lactate even in the presence of oxygen.[3] Despite the controversy on the relation between aerobic glycolysis and cancer biology[4,5] the widespread clinical use of positron-emission tomography (PET) for the detection of aerobic glycolysis in tumors and recent findings have rekindled interest in physiological changes during malignant conversion and metabolic signatures for different stages of tumorigenesis. Although an increase in glucose uptake and lactate production have been correlated to tumor progression, the fully transformed state is most dependent on aerobic glycolysis and almost not on the mitochondrial machinery for ATP synthesis.[2] Thus, aerobic glycolysis can be conceived as a form of tumor adaptation for conditions of reduced or inefficient oxygen supply.

Although the biochemical and molecular mechanisms leading to increased aerobic glycolysis in tumors are complex and may be attributed to multiple factors such as mitochondrial dysfunction, hypoxia and oncogenic signaling, the metabolic consequences seem similar: malignant cells become addicted to glycolysis and dependent on this pathway to generate ATP. Because ATP generation *via* glycolysis is far less efficient than by oxidative phosphorylation (2 *vs*. 36 mol ATP per mol glucose), cancer cells consume far more glucose than normal cells to maintain sufficient ATP supply for their active metabolism and proliferation. As such, maintaining a high-level of glycolytic activity is essential for cancer cells for survival and growth.

Recently, a new link between enhanced glucose consumption and cancer has been described. The transketolase-like protein1 (TKTL1),[6] which has an important role in controlling the nonoxidative pentose-phosphate-pathway (PPP) is significantly overexpressed in several different tumor entities.[7] In addition, increased TKTL1 expression correlates with poor patient outcome and tumor progression.[8–12]

Besides inefficient ATP regeneration, the PPP provides essential NADPH redox equivalents and pentoses for rapidly proliferating cells. This feature may provide a significant advantage to developing and progressing tumors: pentoses are urgently needed for RNA and DNA synthesis and access to NADPH redox equivalents may be essential for tumor cell survival which are challenged by endogenous (*e.g.,* through dysfunctional mitochondria) and exogenous (through immune cells) reactive oxygen species (ROS). These metabolic features have led to the hypothesis that inhibition of glycolysis may severely abolish ATP, ribose and NADPH generation in cancer cells and thus may preferentially kill the malignant cells.[7,10,12–14] In fact, inhibitors of TKTs activity[15] or gene expression[16,17] suppress tumor growth, whereas TKT activation led to enhanced tumor growth.[18] In addition, dietary studies indicate that the inhibition of the PPP suppresses tumor growth.[19–27]

Therapeutic selectivity by preferential killing cancer cells without significant toxicity to normal cells is one of the most important considerations in cancer therapy. Understanding the biological differences between normal and cancer cells is essential for the design and development of such selective anticancer drugs. Because aerobic glycolysis is ubiquitously seen in various cancers, targeting this pathway may have broad implications for therapy.

Here we show that TKTL1 which is specifically overexpressed in malignant cells and tissues, plays important roles in supporting tumor cell growth and increasing tolerance to oxidative stress. Because of its importance for the tumors and its specific expression pattern, TKTL1 may be thus a very promising antitumor target for clinical treatment.

Material and methods

Cell lines, culture conditions and chemicals

Low passage HCT116 cells (ATCC CCL 247) and shRNA-treated cell clones shown to be mycoplasma-free by multiplex

Conflict of Interest: Dr. J. F. Coy declares a potential conflict of interest because of the possible utilization of TKTL1 for diagnostic and/or therapeutic purposes.

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***Correspondence to:** Abt. Genommodifikationen und Karzinogenese, Forschungsprogramm Infektion und Krebs, Deutsches Krebsforschungszentrum (DKFZ), Im Neuenheimer Feld 242, Heidelberg 69120, Germany. Fax: +49-622-142-4932. E-mail: m.loechelt@dkfz.de

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TABLE I – siRNA OLIGONUCLEOTIDES

siRNA names	5′end on mRNA[1]	Target sequence
TKTL1-1	1,406	ACC AGA AAC TAT GGT TAT TTA
TKTL1-2	2,175	AAG TGT TTC CTT CGT GAA TAA
TKTL1-3	560	CGA GCA CTG CAT AAA CAT CTA
TKT	193	AAT CCG CAC AAT GAC CGC TTT

[1]Human TKTL1 and TKT mRNA sequences as taken from accession numbers: NM_012253 and NM_001064, respectively.

PCR tests were maintained as mono-layers in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C and 5% CO_2 (all cell culture reagents purchased from Invitrogen, Karlsruhe, Germany). Cells were trypsinized and passaged 1–2 times a week.

Knockdown experiments by siRNA transfection

HCT116 cells were transfected with a control siRNA (targeting TKT) or 3 siRNA for TKTL1 (Table I, Qiagen, Hilden, Germany) using Lipofectamine 2000 as indicated by the supplier (Invitrogen). TKTL1 transcripts were detected by reverse transcriptase PCR using primer sets 5′GCTGAACAAAACATGGTGAGC-3′and 5′-ACATCCCCTTGGCATTGGCT-3′, β-actin mRNA was amplified in parallel as control[28] with primer sets 5′-ACAAT GAGCTGCGTGTGGCT-3′ and 5′-TCTCCTTAATGTCACG CACGA-3′.

Construction of shRNA expression vectors

Each 3 μg of forward primer 5′-GATCCCCCAGAAAC TATGGTTATTTATTCAAGAGA**TAAATAACCATAGTTTCT GTTTTTGGAAA**-3′ and reverse primer 5′-AGCTTTTCCAAA AACAGAAACTATGGTTATTTATCTCTTGAA**TAAATAACCA TAGTTTCTG**GGG-3′ (5′ and 3′ stem sequences underlined and in bold face letters, respectively) were denatured at 95°C for 4 min, annealed at 70°C for 10 min and slowly cooled down to 4°C in 50 μl annealing buffer (100 mM K-acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM Mg-acetate). The double-stranded sequences were then ligated into BglII- and HindIII-digested pSUPER.neo.GFP. After transforming bacteria, positive clones were selected and verified by DNA sequencing.

Cell transfection and G418 selection

Each 1.5×10^5 HCT116 cells were seeded in 12-well plates in 500 μl medium without antibiotics. Transfections using 2 μl Lipofectamine 2000 and 400 ng plasmid DNA per well were done as recommended by the supplier (Invitrogen). Forty-eight hours after transfection, cells were transferred into 15-cm dishes selected for 14 days in medium containing 1 mg/ml G418 (Sigma, Taufkirchen, Germany). Neo^R cells were subcloned into 6-well plates and maintained in medium with 500 μg/ml G418. Unless otherwise stated, all other reagents were purchased from Sigma.

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using Trizol® (Invitrogen) according to manufacturer's instructions. When DNAse treatment was required, 0.5 μg total RNA was incubated with 2 U RNAse free-DNAse I in DNAse I reaction buffer (New England Biolabs, Frankfurt, Germany) at 37°C for 30 min. The DNAse was inactivated by addition of EDTA ad 5 mM and subsequent incubation at 75°C for 10 min. First strand cDNA was synthesized with Superscript II reverse transcriptase (200 U, Invitrogen) for 1 hr at 42°C using oligo $(dT)_{18}$ primers (Fermentas). Subsequently, the enzyme was inactivated at 90°C for 5 min. To determine changes in TKTL1 expression, qRT-PCR was performed under the following cycling conditions: 10 min at 95°C, then 45 cycles of 95°C for 15 sec and 60°C for 1 min in the ABI Prism 7500 using Power SYBR Green MasterMix (Applied Biosystems, Foster City, CA) and the following primers pairs: hTKTL1 sense 5′-AAGCCTTTGGGTG GAACACTTA-3′, antisense 5′-CTGAGAAGCCTGCCAGAA-TACC-3′; TKT sense 5′-TGTGTCCAGTGCAGTAGTGG-3′, antisense 5′-ACACTTCATACCCGCCC TAG-3′. As endogenous control, β-actin expression was determined using sense and antisense primers 5′-ACTCTTCCAGCCTTCCTTC-3′ and 5′-ATCTCCTTC TGCATCCT GTC-3′. Relative quantification was performed using the δδCt method to determine fold-difference in shRNA transfected HCT116 cells relative to the controls.[29]

D-Glucose consumption and L-Lactic acid production assay

Each 4×10^4 HCT116 stable cell clones with shRNA constructs were plated in 12-well plates in 500 μl medium without antibiotics. Twelve hours after plating, the medium was changed and harvested 24 hr later. To account for differences in cell growth and viability, cell numbers were determined at the time of plating and harvest using a hemocytometer. Glucose metabolism and lactic acid production were normalized to the mean cell number [mean cell number: (cell number plated + cell number harvested)/2]. The concentration of D-Glucose and L-Lactic acid was determined using D-Glucose and Lactate acid detection kits (r-biopharm, Darmstadt, Germany), the consumed D-Glucose and produced L-Lactic acid were calculated accordingly.

Measurement of intracellular ROS

HCT116 cells grown in 12-well plates were incubated at 37°C for 1 hr with fresh RPMI 1640 medium containing 10% FCS and 1% penicillin/streptomycin. Intracellular oxidative stress was monitored by measuring changes in oxidation-dependent fluorescence of the 2 intracellular probes used at the indicated time points. The probe 2′,7′-dichlorofluorescin di-acetate (DCFH-DA, 5 μM, Molecular Probes, Eugene, OR) enters cells and the acetate group on DCFH-DA is cleaved by cellular esterases, trapping the nonfluorescent 2′,7′-dichlorofluorescin (DCFH) intracellularly. Subsequent oxidation by ROS, particularly hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO\cdot$), yields the fluorescent product DCF. Thus, increases in DCF fluorescence are indicative of H_2O_2 or $HO\cdot$ generation. Dihydroethidine (5 μM, Molecular Probes) enters cells and can be oxidized by superoxide (O_2^-) and/or $HO\cdot$ to yield fluorescent ethidium (Eth). Eth binds to DNA (Eth-DNA), further amplifying its fluorescence. Thus, increased nuclear Eth-DNA fluorescence indicates the accumulation of O_2^-/HO^-.

Cell cycle and apoptosis analysis

For quantification of cell cycle progression and the amount of sub-G1 apoptotic cells, HCT116 cells were detached by trypsin treatment and collected by centrifugation. After resuspending in PBS, cells were fixed in 70% ethanol, washed in PBS, stained with 50 μg/ml propidiumiodide containing 50 μg/ml RNAse A in PBS, and analyzed by flow cytometry (FACS-Calibur, Becton Dickenson, Heidelberg, Germany). FACS results were analyzed with Flowjo software.

GSH and NADPH/$NADP^+$ ratio measurement

About 5×10^3 cells per well were plated in 96-well plates. After overnight culture, the medium was removed and cells were washed twice with PBS. Cell lysates were prepared by 2 repetitive freeze-thaw cycles at −80°C (20 min) and 37°C. The cell lysates were resuspended in 125 mM Na_2HPO_4, 6.3 mM EDTA, pH 7.5 and mixed by gentle pipetting. For each reaction, 40 μl of the lysate was transferred to a 96-well plate, GSH standard samples of 0, 0.5, 1, 2, 5, 10, 15, 20 μM were prepared for standard curve determination. The reaction was started by addition of 170 μl of a reaction solution (14.56 ml double distilled H_2O, 770 μl, 0.5 M Tris/HCl, pH 7.5, 102.6 μl 150 mM glucose-6-phosphate, 9.24 μl 50 mM $NADP^+$, 2.5 ml 6 mM DTNB plus 30 U glucose-6-phosphate-dehydrogenase and 30 U glutathione-reductase). The absorbance at 412 nm was measured for 5 min (each 15 sec one time point) at 37°C in a SpectraMax 340PC[384] machine (Molecular

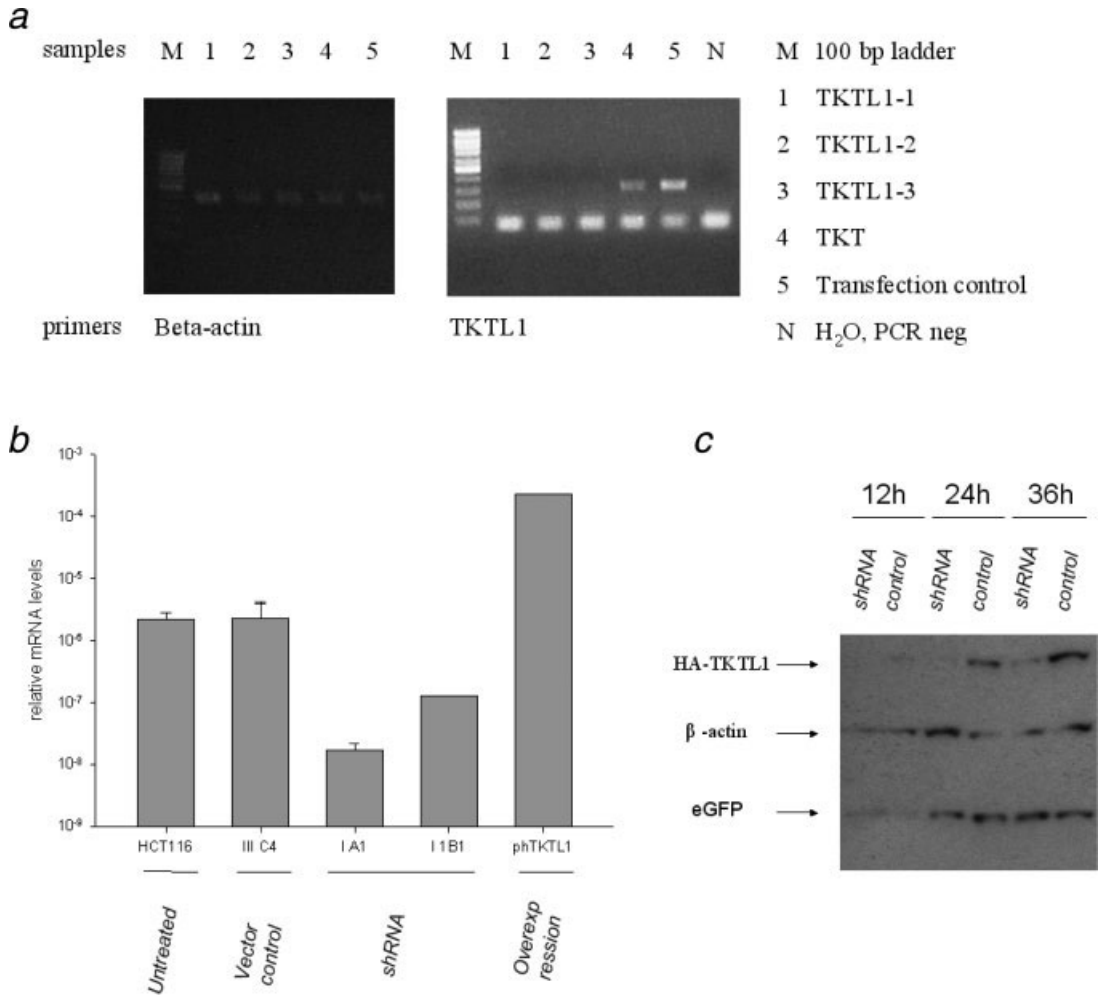

FIGURE 1 – Suppression of TKTL1 by RNA interference. (*a*) HCT116 cells were transiently transfected withTKTL1 siRNA oligos 1, 2 and 3 as shown in Table I. siRNA 4 targets the TKT mRNA whereas the minus sign (−) represents mock-transfected cells. Semiquantitative RT-PCR for TKTL1 and β-actin mRNAs were performed and the products analyzed by gel electrophoresis. The PCR negative control is labeled H_2O and M represents the size marker lane. TKTL1 siRNA oligos 1, 2 and 3 lead to the knockdown of TKTL1 mRNA. An unspecific low-molecular weight band probably corresponding to primer dimers was consistently seen in all TKTL1-specific PCRs (right-hand panel). (*b*) TKTL1 expression was analyzed by real-time PCR in shRNA-treated cell clones IA1 and I1B1 and compared to untreated und vector-treated cells (clone IIIC4) and cells transiently overexpressing TKTL1. Vector control cell clones showed similar expression level of TKTL1 as untreated HCT116 cells. TKTL1-1 shRNA transfected cell clones showed 10- to 100-fold suppressed TKTL1 gene expression. (*c*) TKTL1 shRNA-treated and vector control cell clones were transfected with plasmids expressing HA-tagged TKTL1 and EGFP proteins. TKTL1 was detected by an anti-HA antibody, β-actin and EGPF were detected with corresponding antibodies and served as loading and transfection controls. After 24- and 36-hr transfection, much less TKTL1 band was detected in TKTL1 shRNA-treated cell clones than in the corresponding controls.

Devices, Sunnyvale, CA). Protein concentration was measured with Bradford assay. A series of BSA samples were used for generating a standard curve. The GSH content was calculated as GSH concentration by protein amount.

NADPH and $NADP^+$ concentrations were measured using a kit from Bioassay and the NADPH/$NADP^+$ ratio was calculated using the values measured from the same sample.

Results

siRNA-Mediated suppression of TKTL1 expression

To suppress expression of the endogenous TKTL1 in human cells, siRNA target sequences were identified by an online algorithm (Qiagen). The 3 most promising siRNAs were selected and assayed in cell cultures for their effect on endogenous TKTL1 expression. The positions of the siRNA target sequences on the TKTL1 mRNA and their sequences are given in Table I and Figure S1 (Supp. Info.). The siRNAs were transfected into human colon carcinoma cells HCT116 using Lipofectamine 2000. Two days later, cells were harvested and mRNAs extracted and subjected to semiquantitative reverse transcription PCR. In parallel, the expression level of β-actin mRNA was analyzed and used to normalize the expression level. In repeated experiments, siRNAs 1 and 2 showed the strongest suppression of TKTL1 mRNA whereas siRNAs #3 was less efficient (RT-PCR, Fig. 1*a*). The siRNA 4 targeting the TKT housekeeping gene significantly suppressed TKT mRNA levels but did not affect the highly related TKTL1 (Fig. 1*a*).

shRNA-Mediated suppression of TKTL1 expression

Because siRNAs allow only a transient suppression of targeted genes, we analyzed whether the identified active RNAi target sequences 1 and 2 are also targets of shRNAs allowing long-term suppression of TKTL1 gene expression. For this purpose, shRNA sense and antisense oligonucleotides comprising target sequences 1 and 2 were annealed and inserted into the H1-promoter driven

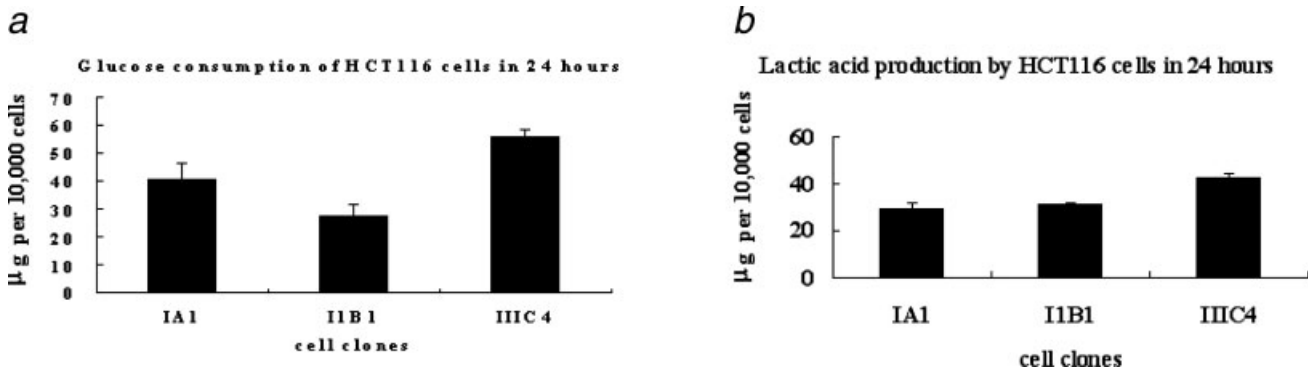

FIGURE 2 – Metabolic features of TKTL1-suppressed HCT116 cells. About 4.0×10^4 TKTL1 (clones IA1 and I1B1) and control shRNA (clone IIIC4) carrying HCT116 cells were plated in 24-well plate supplied with 0.5 ml medium. The culture medium was collected for analysis 24 h after an additional medium exchange and cells were typsinized and counted using a hemocytometer to determine the overall cell numbers. Cell culture supernatants were assayed for D-glucose consumption (*a*) and L-lactic acid production (*b*), the corresponding values are normalized to the mean cell numbers.

shRNA expression plasmid pSUPER.neo.GFP. Besides directing shRNA expression, the vector encodes neomycin (G418) resistance allowing selection of stable single-cell clones, a technique absolutely required for long-term studies of growth-attenuated cells.

To assess the efficacy of the constructs, both TKTL1 shRNAs and the empty control vector were transfected into human HCT116 cells and stable single-cell clones were selected in G418-containing medium. According to a quantitative RT-PCR (qRT-PCR) protocol established in the lab (see "Material and methods" section), TKTL1 expression in untreated or control empty vector-treated cells was up to 50-fold higher than in bulk cell cultures treated with shRNA-I targeting TKTL1 mRNA residues 1406–1426. Independent TKTL1 shRNA-I cell clones (*e.g.,* IA1, I1B1) displayed clonal variation with respect to TKTL1 expression between a 5-fold and more than 100-fold mRNA suppression (Fig. 1*b*) relative to empty vector-treated cell clones IIIC4 and IIIA2. In contrast, shRNA-II (targeting TKTL1 mRNA residues 2175–2195 in the 3′-untranslated region) suppressed TKTL1 mRNA levels only marginally (about 10% suppression). Consequently, all subsequent studies were done using TKTL1 shRNA-I.

Using the TKTL1-specific monoclonal antibody described previously,[30] we were not able to consistently detect endogenous TKTL1 protein in HCT116 cells and hence could not detect the shRNA-mediated TKTL1 protein suppression directly. To circumvent this limitation, we transfected established and well-characterized TKTL1 shRNA-I cell clones and untreated HCT116 cells with HA-tagged hTKTL1 overexpression plasmid. Subsequent immunoblotting using the high-affinity anti-HA monoclonal antibody targeting the HA-TKTL1 fusion protein revealed a clear repression of TKTL1 protein expression in shRNA-I-containing cell clones compared with the controls (Fig. 1*c*). To compensate for experimental variations, TKTL1 expression was pair-wise normalized to the β-actin loading control and cotransfected Gfp by densitometry of the immunoblots (data not shown). Using both standards, significant and strong TKTL1 suppression was confirmed in TKTL1 shRNA-treated cells 24 and 36 hr after transfection demonstrating that TKTL1 knockdown cell clones had been successfully established.

Altered glucose metabolism by shRNA-mediated TKTL suppression

TKTL1 is considered to play a central role in glucose metabolism and aerobic glycolysis directly enhancing glucose consumption and lactic acid release.[30] Thus, we first studied whether suppression of TKTL1 expression results in altered glucose metabolism or lactate production. For this purpose, cell clones with repressed TKTL1 expression (IA1, I1B1) and control cell clones (IIIC4) were assayed for D-Glucose consumption and L-Lactic acid production. Identical amounts of cells were cultured (see "Material and methods" section) and glucose consumption and lactate production were determined for a 24-hr interval (12–36 hr after splitting). To account for differences in cell growth, cell numbers were determined at the time of plating and harvest using an hemocytometer. Glucose metabolism and lactic acid production were normalized to the mean cell number (cell number plated + cell number harvested)/2. We found that TKTL1-suppressed cell clones have reproducibly a reduced D-Glucose consumption rate. Within 24 hr, cell clone I1B1 consumed only half of the D-Glucose compared to control clone IIIC4, whereas shRNA-treated clone IA1 displayed around 30% lower glucose consumption (Fig. 2*a*). The altered glucose metabolism of cells with suppressed TKTL1 expression was paralleled by reduced L-lactic acid production. Within the 24-hr interval, the TKTL1-knockdown clones produced around 30% less L-lactic acid than the control (Fig. 2*b*). Overall, the data are in line with the concept that TKTL1 suppression in HCT116 cells blocks the D-glucose flow into the PPP with the consequence that these cells consume less glucose and thus also produce less lactic acid.

Reduced cell proliferation by shRNA-mediated TKTL1 suppression

As TKTL1 is an important enzyme in the PPP, a pathway which is not only utilized for energy production but also for nucleic acid precursor synthesis and generation of redox equivalents, we studied whether suppression of TKTL1 expression has an effect on cell proliferation. Equal amounts of cells (2×10^5) were plated in 35 mm cell culture dishes with standard culture medium. Cells were harvested 2 days after plating and analyzed for cell cycle distribution by FACS. The data revealed that TKTL1-suppressed HCT116 cell clones had higher G0/G1 but lower S and G2/M phase peaks than the corresponding empty vector-treated cells (Figs. 3*a* and 3*b*). TKTL1-shRNA treated clones IA1 and I1B1 had about 80% of the cells in G0/G1 phase, ~10% cells in S phase and 10% in G2/M phase, whereas in control clone IIIC4, the S phase and G2/M phase cells increased to 18.4 and 20.9%, respectively. Thus, the cell population entering the cell cycle was reduced to half of the control cells upon TKTL1 suppression. In line with this finding, cell growth kinetics showed that TKTL1-suppressed cells grew much slower than controls (Fig. 3*c*). For these experiments, 4×10^4 cells per well were seeded in 12-well plates and left to attach for 12 hr. Cells were harvested and counted using a hemocytometer every 24 hr. The experiment was done in triplicates, both TKTL1 shRNA-treated cell clones showed similar growth kinetics. Within 4 days, the cell number raised from 4×10^4 to around 5×10^5, while control cell numbers

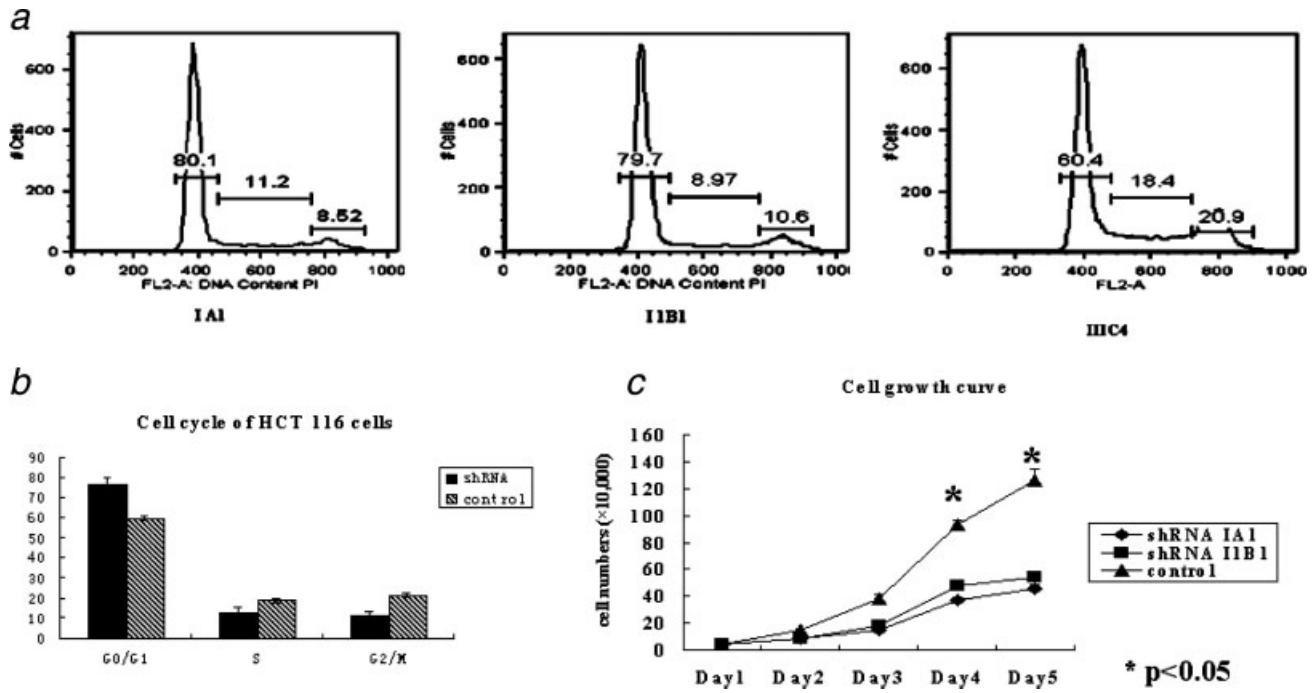

FIGURE 3 – Growth patterns of TKTL1-suppressed HCT116 cells. (*a*) Cell cycle distribution by FACS showed that TKTL1-suppressed clones IA1 and I1B1 displayed reduced cell numbers in the G2/M and S phase of the cell cycle and correspondingly increased numbers of cells in the G0/G1 phase compared to control clone IIIC4. The data of 3 corresponding experiments are summarized in (*b*) Showing that these differences are highly reproducible. (*c*) Cell growth curves were generated for the different HCT116 clones showing that TKTL1-suppressed clones IA1 and I1B1 grow significantly (*) slower compared to the control clone IIIC4. After 4 days, TKTL1 suppression led to 2-fold reduced cell growth compared to the control clone.

reached, on average, 1.3×10^6, more than twice the number of TKTL1-shRNA treated cells. These data indicate that TKTL1 suppression leads to cell cycle arrest in the G0/G1 phase of the cell cycle indicating that TKTL1 promotes cell cycle progression and proliferation.

TKTL1-Suppressed cells display enhanced sensitivity towards oxidative stress

The PPP which is controlled by TKT activity provides essential redox equivalents for reductive biosynthesis and detoxification of highly ROS. For this reason, we analyzed whether TKTL1 down-regulation leads to higher intracellular ROS levels. In these experiments, intracellular ROS were stained with fluorescence dyes and detected by FACS. As shown in Figure 4, the intracellular levels superoxide anion (stained with DHE), were not significantly increased in TKTL1 knockdown clone I1B1 compared to control clone IIIA2, whereas H_2O_2 (stained with DCFDA) was slightly increased in TKTL1 knockdown clone I1B1 under standard cell culture conditions (Figs. 4*a* and 4*b*). Concomitantly, TKTL1-suppressed cells displayed increased marker of apoptosis as indicated by the appearance of morphological alterations such as cell shrinkage and nuclear condensation (Supp. Info. Fig. S2). Most importantly, TKTL1-suppressed cells displayed an increased number of sub-G0/G1 cells in FACS analyses when subjected to oxidative stress by the addition of H_2O_2 and PEITC for 24 hr (Figs. 4*c* and 4*d*).

The NADPH/$NADP^+$ ratio and the relative amount of the reduced form of glutathione (GSH) represent an established readout of the redox status of the cells, because NADPH and GSH are major redox equivalent to counteract oxidative stress inside the cells.[32,33] Because the PPP produces NADPH, it is conceivable that upon TKTL1 suppression, intracellular NADPH and GSH concentrations are reduced, leading to a decreased NADPH/$NADP^+$ ratio and a lower GSH content. The results show that the anti-ROS defense system in TKTL1-suppressed cells is much weaker than in control cells grown under comparable conditions (Fig. 5). Taken together, TKTL1 seems to have a protective effect against oxidative or radical stress.

Discussion

Cell immortalization and malignant transformation are mainly determined by genetically altered genes or their aberrant expression.[34] Genetic alterations acquired by tumor cells also modify their biochemical pathways to support tumor growth, viability, and spread. Tumors normally have abnormal metabolism properties owing to the abnormal growth. Aerobic glycolysis (Warburg effect) is a very common metabolic feature of tumors, although the molecular basis has not been identified yet. However, the biological consequence of the aerobic glycolysis, *i.e.* the enhanced metabolism of glucose, is already successfully applied in the clinic by the PET technique to visualize tumors and metastases.[35,36]

Recent studies showed that TKTL1 which is characterized by distinct enzymatic features[30] is specifically overexpressed in tumors predicting poor cancer patient survival.[7] In contrast, TKT and TKTL2 expression was not altered, suggesting that TKTL1 plays an important role in tumor-specific glucose metabolism.[7] When TKTL1 expression is suppressed by siRNA or shRNA constructs as done in this and other studies,[16,17] the TKTL1-suppressed cells display significantly decreased growth and proliferation rates. This may be in part due to the fact that ATP and/or glucose supply of these treated cells has been down-regulated. In addition, it is possible that end products of the hexose/pentose pathways, which may accumulate upon TKTL1 suppression have negative effects on cell viability and growth, for instance by enhanced glycation of cellular proteins.

In our article, we experimentally address additional phenotypic consequences of the suppressed TKTL1 expression. We show that

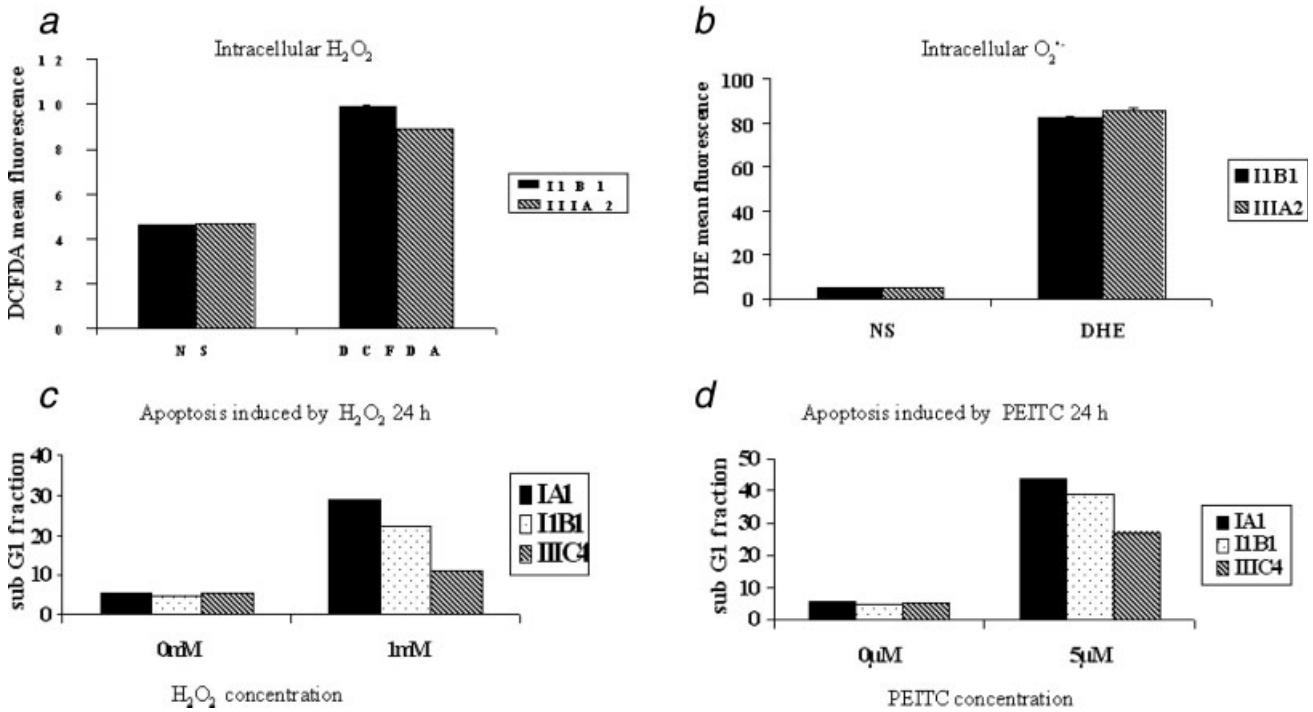

Figure 4 – Intracellular ROS levels and sensitivity towards ROS-induced apoptosis in TKTL1-suppressed cells. Compared to control cell clone IIIA2, TKTL1-suppressed clone I1B1 displayed slightly increased intracellular H_2O_2 levels (*a*) but no significant change in the O_2^- content (*b*). When the different HCT116 cell clones were challenged with either H_2O_2 (*c*) or PEITC which increase H_2O_2 levels in cancer cells[31] (*d*), TKTL1-suppressed cells showed increased sensitivity towards ROS-induced apoptosis as measured by the fraction of sub G1 cells in FACS analyses.

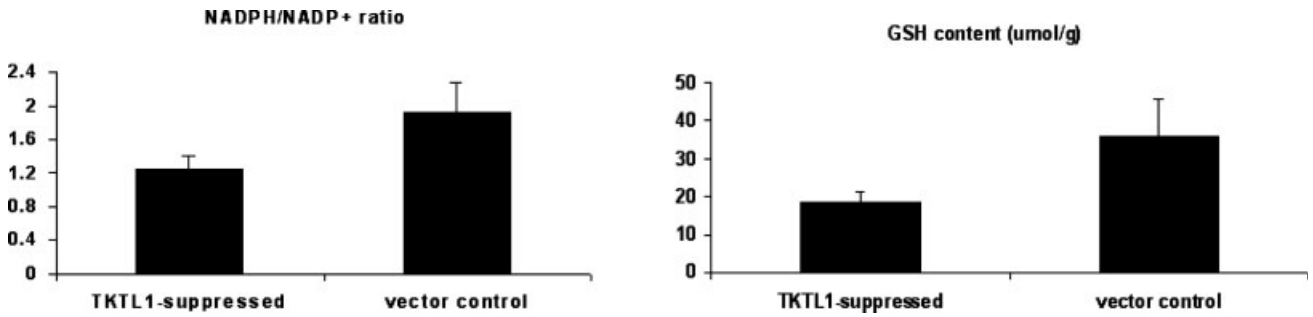

Figure 5 – Decreased concentrations of redox equivalents in TKTL1-suppressed cells. In TKTL1-suppressed HCT116 cells (clone IA1), the ratio of reduced *vs*. oxidized $NADP^+$ and the content of reduced GSH were both clearly suppressed in comparison to the shRNA vector control (clone IIIC4) indicating that TKTL1 suppression weakens the cellular anti-ROS defense systems.

both, a lower glucose consumption and L-lactic acid production rate are induced by TKTL1 suppression as anticipated from the assumed role of TKTL1 as major regulator of PPP in tumor cells[30] although we can not formally exclude the possibility that these metabolic changes are only indirectly affected by TKTL1 suppression. In line with direct effects of TKTL1 on glucose metabolism (and lactate production), recent studies utilizing radioactively labeled glucose have revealed that glucose, besides its central role in energy production, is the main source of tumor cell RNA and DNA pentoses[37,38] and *de novo* lipid synthesis.[39] The strong proliferation process of tumor cells is governed by the replication of DNA in the S phase where the conversion of glucose to ribose is controlled by nonoxidative PPP. It is thus not surprising that TKTL1-suppressed cells showed slower growth rates and cell cycle arrested in G0/G1 phase as observed by flow cytometry (Fig. 3). Since lactate concentration correlates with metastases and poor patient survival,[40,41] blocking TKTL1 activity may thus reduce tumor invasiveness and could achieve a better patient's prognosis.

Another important and common biochemical change in cancer cells is the increase in hazardous ROS which are detrimental for cell survival but which may themselves promote further tumor progression[42]: Increased ROS levels are thought to play an important role in maintaining or promoting the cancer phenotype because of their stimulating effects on cell growth and proliferation,[43] genetic instability[44] and senescence evasion.[45] However, high levels of ROS can also cause cellular damage, depending on the levels and duration of ROS stress.[46] A classical function of PPP in mammalian cells is to provide NADPH redox equivalents through carbon flow from C_6 sugars (hexoses) to C_5 sugars (pentoses). As NADPH is one of the most important metabolites to maintain the intracellular redox balance, blocking PPP by TKTL1 suppression can be predicted to disturb this balance thus increasing the susceptibility of the affected cell towards oxidative stress. Although the basal ROS levels and apoptosis rates of TKTL1-suppressed and control cells were similar under standard cell culture conditions except the slightly increase in intracellular H_2O_2

(Figs. 4*a* and 4*b*), additional extracellular oxidative stress by exogenous H_2O_2, or intracellular stress through PEITC (generating ROS inside cells) in fact increased ROS and apoptosis levels overproportionally in TKTL1-suppressed cells. This indicates that tumor cells may clearly benefit from high-level expression of TKTL1 when facing oxidative stress, which is commonly occurring in fast growing tumors.

In summary, our data presented here show the strong involvement of TKTL1 in cancer cell growth and viability. Because of its specific overexpression in tumor and malignant cells,[30] TKTL1 may be thus not only considered as a tumor marker but also as a strong candidate target for anticancer therapy. We are pursuing *in vivo* experiments to verify this hypothesis.

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References

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100: 57–70.
2. Ramanathan A, Wang C, Schreiber SL. Perturbational profiling of a cell-line model of tumorigenesis by using metabolic measurements. Proc Natl Acad Sci USA 2005;102:5992–7.
3. Warburg O, Posener K, Negelein E. Über den stoffwechsel der carcinomzelle. Biochem Z 1924;152:309–44.
4. Garber K. Energy boost: the Warburg effect returns in a new theory of cancer. J Natl Cancer Inst 2004;96:1805–6.
5. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 2004;4:891–9.
6. Coy JF, Dübel S, Kioschis P, Thomas K, Micklem G, Delius H, Poustka A. Molecular cloning of tissue-specific transcripts of a transketolase-related gene: implications for the evolution of new vertebrate genes. Genomics 1996;32:309–16.
7. Langbein S, Zerilli M, Zur Hausen A, Staiger W, Rensch-Boschert K, Lukan N, Popa J, Ternullo MP, Steidler A, Weiss C, Grobholz R, Willeke F, et al. Expression of transketolase TKTL1 predicts colon and urothelial cancer patient survival: warburg effect reinterpreted. Br J Cancer 2006;94:578–85.
8. Zerilli M, Amato MC, Martorana A, Cabibi D, Coy JF, Cappello F, Pompei G, Russo A, Giordano C, Rodolico V. Increased expression of transketolase-like-1 in papillary thyroid carcinomas smaller than 1.5 cm in diameter is associated with lymph-node metastases. Cancer 2008;113:936–44.
9. Voelker HU, Hagemann C, Coy J, Wittig R, Sommer S, Stojic J, Haubitz I, Vince GH, Kaemmerer U, Monoranu CM. Expression of transketolase-like 1 and activation of Akt in grade IV glioblastomas compared with grades II and III astrocytic gliomas. Am J Clin Pathol 2008;130:50–7.
10. Langbein S, Frederiks WM, zur Hausen A, Popa J, Lehmann J, Weiss C, Alken P, Coy JF. Metastasis is promoted by a bioenergetic switch: new targets for progressive renal cell cancer. Int J Cancer 2008;122:2422–8.
11. Zhang S, Yue JX, Yang JH, Cai PC, Kong WJ. Overexpression of transketolase protein TKTL1 is associated with occurrence and progression in nasopharyngeal carcinoma: a potential therapeutic target in nasopharyngeal carcinoma. Cancer Biol Ther 2008;7:517–22.
12. Wittig R, Coy JF. The role of glucose metabolism and glucose-associated signalling in cancer. Perspect Med Chem 2007;1:64–82.
13. Munoz-Pinedo C, Ruiz-Ruiz C, Ruiz de Almodovar C, Palacios C, Lopez-Rivas A. Inhibition of glucose metabolism sensitizes tumor cells to death receptor-triggered apoptosis through enhancement of death-inducing signaling complex formation and apical procaspase-8 processing. J Biol Chem 2003;278:12759–68.
14. Xu RH, Pelicano H, Zhou Y, Carew JS, Feng L, Bhalla KN, Keating MJ, Huang P. Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res 2005;65:613–21.
15. Rais B, Comin B, Puigjaner J, Brandes JL, Creppy E, Saboureau D, Ennamany R, Lee WN, Boros LG, Cascante M. Oxythiamine and dehydroepiandrosterone induce a G1 phase cycle arrest in Ehrlich's tumor cells through inhibition of the pentose cycle. FEBS Lett 1999;456:113–8.
16. Hu LH, Yang JH, Zhang DT, Zhang S, Wang L, Cai PC, Zheng JF, Huang JS. The TKTL1 gene influences total transketolase activity and cell proliferation in human colon cancer LoVo cells. Anticancer Drugs 2007;18:427–33.
17. Zhang S, Yang JH, Guo CK, Cai PC. Gene silencing of TKTL1 by RNAi inhibits cell proliferation in human hepatoma cells. Cancer Lett 2007;253:108–14.
18. Comin-Anduix B, Boren J, Martinez S, Moro C, Centelles JJ, Trebukhina R, Petushok N, Lee WN, Boros LG, Cascante M. The effect of thiamine supplementation on tumour proliferation. A metabolic control analysis study. Eur J Biochem 2001;268:4177–82.
19. Ashrafian H. Cancer's sweet tooth: the Janus effect of glucose metabolism in tumorigenesis. Lancet 2006;367:618–21.
20. Boros LG, Bassilian S, Lim S, Lee WN. Genistein inhibits nonoxidative ribose synthesis in MIA pancreatic adenocarcinoma cells: a new mechanism of controlling tumor growth. Pancreas 2001;22:1–7.
21. Boros LG, Lapis K, Szende B, Tomoskozi-Farkas R, Balogh A, Boren J, Marin S, Cascante M, Hidvegi M. Wheat germ extract decreases glucose uptake and RNA ribose formation but increases fatty acid synthesis in MIA pancreatic adenocarcinoma cells. Pancreas 2001;23:141–7.
22. Comin-Anduix B, Boros LG, Marin S, Boren J, Callol-Massot C, Centelles JJ, Torres JL, Agell N, Bassilian S, Cascante M. Fermented wheat germ extract inhibits glycolysis/pentose cycle enzymes and induces apoptosis through poly(ADP-ribose) polymerase activation in Jurkat T-cell leukemia tumor cells. J Biol Chem 2002;277:46408–14.
23. Hidvegi M, Raso E, Tomoskozi-Farkas R, Szende B, Paku S, Pronai L, Bocsi J, Lapis K. MSC, a new benzoquinone-containing natural product with antimetastatic effect. Cancer Biother Radiopharm 1999; 14:277–89.
24. Jakab F, Shoenfeld Y, Balogh A, Nichelatti M, Hoffmann A, Kahan Z, Lapis K, Mayer A, Sapy P, Szentpetery F, Telekes A, Thurzo L, et al. A medical nutriment has supportive value in the treatment of colorectal cancer. Br J Cancer 2003;89:465–9.
25. Nebeling LC, Lerner E. Implementing a ketogenic diet based on medium-chain triglyceride oil in pediatric patients with cancer. J Am Diet Assoc 1995;95:693–7.
26. Seyfried TN, Sanderson TM, El-Abbadi MM, McGowan R, Mukherjee P. Role of glucose and ketone bodies in the metabolic control of experimental brain cancer. Br J Cancer 2003;89:1375–82.
27. Otto C, Kaemmerer U, Illert B, Muehling B, Pfetzer N, Wittig R, Voelker HU, Thiede A, Coy JF. Growth of human gastric cancer cells in nude mice is delayed by a ketogenic diet supplemented with omega-3 fatty acids and medium-chain triglycerides. BMC Cancer 2008;30:122.
28. Yousef GM, Obiezu CV, Luo LY, Black MH, Diamandis EP. Prostase/KLK-L1 is a new member of the human kallikrein gene family, is expressed in prostate and breast tissues, and is hormonally regulated. Cancer Res 1999;59:4252–6.
29. Makedonski K, Abuhatzira L, Kaufman Y, Razin A, Shemer R. MeCP2 deficiency in Rett syndrome causes epigenetic aberrations at the PWS/AS imprinting center that affects UBE3A expression. Hum Mol Genet 2005;14:1049–58.
30. Coy JF, Dressler D, Wilde J, Schubert P. Mutations in the transketolase-like gene TKTL1: clinical implications for neurodegenerative diseases, diabetes and cancer. Clin Lab 2005;51:257–73.
31. Zhang Y, Tang L, Gonzalez V. Selected isothiocyanates rapidly induce growth inhibition of cancer cells. Mol Cancer Ther 2003;2: 1045–52.
32. Kirsch M, De Groot H. NAD(P)H, a directly operating antioxidant? FASEB J 2001;15:1569–74.
33. Pastore A, Federici G, Bertini E, Piemonte F. Analysis of glutathione: implication in redox and detoxification. Clin Chim Acta 2003;333: 19–39.
34. Weng G, Bhalla US, Iyengar R. Complexity in biological signaling systems. Science 1999;284:92–6.
35. Downey RJ, Akhurst T, Gonen M, Vincent A, Bains MS, Larson S, Rusch V. Preoperative F-18 fluorodeoxyglucose-positron emission tomography maximal standardized uptake value predicts survival after lung cancer resection. J Clin Oncol 2004;22:3255–60.
36. Kumar R, Xiu Y, Mavi A, El-Haddad G, Zhuang H, Alavi A. FDG-PET imaging in primary bilateral adrenal lymphoma: a case report and review of the literature. Clin Nucl Med 2005;30:222–30.
37. Boros LG, Puigjaner J, Cascante M, Lee WN, Brandes JL, Bassilian S, Yusuf FI, Williams RD, Muscarella P, Melvin WS, Schirmer WJ. Oxythiamine and dehydroepiandrosterone inhibit the nonoxidative synthesis of ribose and tumor cell proliferation. Cancer Res 1997;57: 4242–8.
38. Horecker BL, Domagk G, Hiatt HH. A comparison of C14-labeling patterns in deoxyribose and ribose in mammalian cells. Arch Biochem Biophys 1958;78:510–7.

39. Lee WN, Byerley LO, Bassilian S, Ajie HO, Clark I, Edmond J, Bergner EA. Isotopomer study of lipogenesis in human hepatoma cells in culture: contribution of carbon and hydrogen atoms from glucose. Anal Biochem 1995;226:100–12.
40. Stern R, Shuster S, Neudecker BA, Formby B. Lactate stimulates fibroblast expression of hyaluronan and CD44: the Warburg effect revisited. Exp Cell Res 2002;276:24–31.
41. Walenta S, Wetterling M, Lehrke M, Schwickert G, Sundfor K, Rofstad EK, Mueller-Klieser W. High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. Cancer Res 2000;60:916–21.
42. Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 1991;51:794–8.
43. Hu Y, Rosen DG, Zhou Y, Feng L, Yang G, Liu J, Huang P. Mitochondrial manganese-superoxide dismutase expression in ovarian cancer: role in cell proliferation and response to oxidative stress. J Biol Chem 2005;280:39485–92.
44. Radisky DC, Levy DD, Littlepage LE, Liu H, Nelson CM, Fata JE, Leake D, Godden EL, Albertson DG, Nieto MA, Werb Z, Bissell MJ. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. Nature 2005;436:123–7.
45. Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, Koutcher JA, Scher HI, Ludwig T, Gerald W, Cordon-Cardo C, Pandolfi PP. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature 2005;436:725–30.
46. Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. Drug Resist Update 2004;7:97–110.