Mutations in the Transketolase-like Gene TKTL1: Clinical Implications for Neurodegenerative Diseases, Diabetes and Cancer

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SUMMARY

Transketolase proteins or transketolase enzyme activities have been related to neurodegenerative diseases, diabetes, and cancer. Transketolase enzyme variants and reduced transketolase enzyme activities are present in patients with the neurodegenerative disease Wernicke-Korsakoff syndrome. In Alzheimer’s disease patients transketolase protein variants with different isoelectric points or a proteolytic cleavage leading to small transketolase protein isoforms have been identified. In diabetes mellitus patients reduced transketolase enzyme activities have been detected and the lipid-soluble thiamine derivative benfotiamine activates transketolase enzyme reactions, thereby blocking three major pathways of hyperglycemic damage and preventing diabetic retinopathy. In cancer inhibition of transketolase enzyme reactions suppresses tumor growth and metastasis. All the observed phenomena have been interpreted solely on the basis of a single transketolase gene (TKT) encoding a single transketolase enzyme. No mutations have been identified so far in TKT transketolase explaining the altered transketolase proteins or transketolase enzyme activities found in neurodegenerative diseases, diabetes and cancer. We demonstrate the presence of a second transketolase enzyme (TKTL1) in humans. During the evolution of the vertebrate genome, mutations in this transketolase gene (TKTL1) have led to tissue-specific transcripts different in size which encode an enzymatically active transketolase protein as well as different smaller protein isoforms. The mutations within the TKTL1 gene caused a mutant transketolase enzyme with an altered substrate specificity and reaction modus. Here we characterize the TKTL1 gene and its encoded TKTL1 protein(s) and discuss the medical and clinical implications of this mutated transketolase. We furthermore postulate a novel metabolic concept for the understanding, prevention and therapy of neurodegenerative diseases, diabetes and cancer.

KEY WORDS

Pentose phosphate pathway, transketolase, neurodegenerative disease, aging, advanced glycation endproduct (AGE), diabetes, cancer, aerobic glycolysis, Warburg effect, glucose metabolism, positron emission tomography (PET)

INTRODUCTION

The F-18 fluorodeoxyglucose ([18F]FDG) positron emission tomography (PET) imaging technology is being used to visualize an altered glucose metabolism in cancer patients and neurodegenerative disease patients. Although the molecular basis for the altered glucose metabolism has not been identified yet, the PET technique is successfully being clinically applied. An enhanced glucose usage is visualized in tumors and metastases, whereas a reduced cerebral glucose metabolism is detected in Alzheimer’s disease (AD) patients, even before the onset of clinical symptoms. Two main biochemical pathways of glucose metabolism have been identified. The observation in the 1930s that muscle extracts can catalyze the glycolysis of glucose to lactate led to the identification of the Embden-Meyerhof pathway. Within this pathway fructose-1,6-diphosphate is cleaved leading to pyruvate, which is reduced to lactate in the absence of oxygen. In addition glucose is also degraded via the pentose phosphate pathway (PPP). The
nonoxidative part of the PPP is controlled by transketolase enzyme reactions. The PPP, also known as the phosphogluconate pathway or the hexose monophosphate shunt, represents a multifunctional pathway. Three main activities have been attributed to this pathway, depending on the cell type and its metabolic state. The first function of the pentose pathway in mammalian cells is the generation of reducing power in the form of NADPH through a carbon flow from hexoses (mainly glucose) to pentoses which are recycled back into glycolysis through the nonoxidative pathway. The second function is the complete oxidative degradation of pentoses by converting them into hexoses, which can enter the glycolytic sequence. The third function of the PPP is to convert hexoses into pentoses, particularly ribose-5-phosphate, required in the synthesis of nucleic acids.

Although the PPP represents a basic biochemical pathway, the proposed reactions of the nonoxidative PPP presented in textbooks is still controversial because the degree of $^{14}$C isotope labelling and its distribution in carbon atoms of fructose-6-phosphate differed from that predicted by reaction sequences\cite{4,5,6,7}. The authors themselves detected this disquieting difference between theory and experimental results. Surprisingly, the notable difference between theory and experimental results received little adverse comment and generally uncritical approval from pentose pathway reviewers and textbook authors from that time. To explain the discrepancy between the proposed reaction sequences and the observed labeling results, a mathematical model has been developed\cite{8}, but no further experimental analysis of this elementary biochemical reaction sequences has been performed, which could explain the observed results. Therefore the current interpretation of results regarding transketolase enzyme reactions are either based on reaction sequences proposed 50 years ago, which are still controversial, or are based on a mathematical model trying to explain these discrepancies between theory and fact.

Furthermore, all transketolase related results have been interpreted solely on the basis of a single transketolase gene (TKT), although a transketolase-like gene (TKTL1) has been identified\cite{9}. Up to now, one transketolase (TKT) and two transketolase-like genes (TKTL1 and TKTL2) have been identified in the human genome. To evaluate the role of the three transketolase genes in diseases, we analyzed in a first step the expression of all members of the TKT gene family in cancer. Although the crucial role of transketolase enzyme reactions for tumorigenesis and metastasis is known, no molecular or immunohistochemical analysis of the three candidate genes has been performed previously. We could demonstrate that TKTL1 is specifically upregulated in malignancies at the mRNA level, whereas TKT and TKTL2 are not upregulated. We confirmed the upregulation of the TKTL1 protein in malignancies also at the protein level. We demonstrated the clinical importance of TKTL1 upregulation, since TKTL1 expression was correlated to invasive colonic and urothelial tumors and to poor patient outcome (Langbein et al., submitted). These findings suggest TKTL1 as the relevant target for novel anti-transketolase cancer therapies.

There is strong evidence that transketolase proteins or transketolase enzyme reactions are also important for diabetes as well as for neurodegenerative diseases. A high blood glucose level leads to severe chronic complications in a subgroup of diabetes patients. Due to a high blood glucose level and a concomitant high glucose concentration in retinal, endothelial and neuronal cells, glucose and other reducing sugars react nonenzymatically with protein amino groups to initiate a post-translational modification process known as nonenzymatic glycation\cite{10,11}. In diabetes patients such an advanced glycation gives rise to advanced glycation end products (AGE), thereby leading to macro- or microvascular complications, neuropathy and retinopathy. Glucose metabolism leading to AGE formation and retinopathy can be altered by application of vitamin B1 (thiamine), a cofactor of transketolase enzymes. The lipid-soluble thiamine derivative benfotiamine activates transketolase enzyme reactions and blocks three major pathways of hyperglycemic damage and prevents diabetic retinopathy\cite{12}. Therefore activation of transketolase enzyme reactions represents a major progress in therapy and prevention of chronic diabetes complications.

Glucose metabolism and the formation of AGE have also been suggested as a model of aging and was also linked to neurodegenerative diseases like AD. A glycation of proteins in senile plaques of AD patients has been detected. Many senile plaques contained glucose-AGE, indicating that Aβ is glycated by glucose\cite{13}. Furthermore, a well-defined risk factor for the onset of AD is possession of one or more alleles of the epsilon-4 variant (E4) of the apolipoprotein E (ApoE) gene. Meta-analysis of allele frequencies has found that E4 is rare in populations with long historical exposure to agriculture, suggesting that consumption of a high carbohydrate diet may have selected against E4 carriers\cite{14}. Furthermore, the role of the ApoE4 allele for a reduced cerebral glucose metabolism in aging as well as for a more severe AD phenotype has been shown by PET\cite{15,16}.

Alterations of enzymes or enzyme activities involved in glucose metabolism have already been identified in neurodegenerative diseases. Transketolase protein variants and reduced transketolase enzyme activities have been detected in Wernicke-Korsakoff syndrome patients\cite{17,18}, and in AD patients transketolase protein variants with different isoelectric points or a proteolytic cleavage leading to small transketolase protein isoforms have been identified\cite{19,20}. Until now, no mutations in Wernicke-Korsakoff syndrome patients have been identified in the TKT transketolase gene\cite{21}.

Here, we characterize the transketolase-like gene TKTL1. Due to a mutation in a putative exon, a stop codon has been detected in the predicted open reading frame. Therefore the TKTL1 gene has been assumed to
be a pseudogene; however, we have previously shown that TKTL1 in fact could encode a transketolase-like protein. Using a novel monoclonal antibody specifically detecting the TKTL1 protein on paraffin sections, and in ELISA and Western blot format, we were able to identify and isolate native TKTL1 protein(s). In contrast to known transketolase genes/proteins, the TKTL1 gene encodes different tissue-specific transcripts and a full length as well as smaller protein isoforms. The TKTL1 gene encodes a transketolase with unusual enzymatic properties, which are likely to be caused by the internal deletion of conserved residues. The unique enzymatic properties of the TKTL1 protein and the presence of smaller TKTL1 protein isoforms could explain the alterations of transketolase enzyme reactions and the observed transketolase protein variants in neurodegenerative diseases. Expression analysis of the TKTL1 gene furthermore indicates that the TKTL1 protein is correlated to a certain type of glucose metabolism (aerobic glycolysis; Warburg effect) and to cells which are affected by chronic complications of diabetic patients. Based on the unusual features of the TKTL1 gene/protein(s), we postulate a novel metabolic pathway as a basis for the understanding, prevention and therapy of invasive cancer, neurodegenerative diseases, aging, cardiovascular diseases and chronic diabetes complications.

RESULTS

Three transketolase(-like) genes are present in the human genome

The TKTL1 gene represents one of three highly similar transketolase(-like) genes in the human genome (TKT, TKTL1, TKTL2). The TKT and the TKTL1 gene share a similar gene structure, whereas the TKTL2 gene is an intronless gene. TKT and TKTL2 transcripts harbor exon 3 sequences or sequences homologous to exon 3. The TKTL1 transcript, however, lacks exon 3 due to an internal deletion. Downstream of this deletion, the sequence identity between TKTL1 and TKT is 66% on the DNA level and 63% on the protein level, similar to values obtained between paralogues of a gene family which arose by genome duplications. In contrast to this, the sequence identity between TKTL1 and TKTL2 is 80% on the DNA level and 77% on the protein level. Orthologues of TKT, TKTL1 and TKTL2 were also identified in mouse and rat. In frogs (Xenopus laevis) and fish (Danio rerio) only a single transketolase gene with no exon 3 deletion has been identified. In mouse and rat three transketolase(-like) genes are present and similar to the human TKTL1 transcript, the TKTL1 orthologous transcript also harbors the exon 3 deletion. The genomic structure of the three transketolase genes and the presence of the exon 3 deletion in TKTL1 in humans, mouse and rat indicate that during evolution of the vertebrate genome, this deletion occurred within the TKTL1 gene before the human and murine lineages diverged. The high similarity between TKTL2 and TKTL1 suggests that prior to the deletion of exon 3, an intact copy of TKTL1 was duplicated and integrated into the genome by a reverse transcriptase-mediated event, giving rise to TKTL2.

Tissue-specific mRNA expression of TKTL1

To examine the expression level of different TKTL1 transcripts, Northern blot hybridization was performed using multiple human adult tissues (Figure 1A). To avoid the possibility that the detected transcripts are due to cross-hybridization with TKT or TKTL2 gene transcripts, we used a probe from the 3' untranslated region, with only limited sequence homologies to TKT and TKTL2. Four distinct species were identified: one of 2.5 kb was detected in all tissues examined with the exception of adult heart. A larger transcript of app. 2.7 kb was weakly expressed in lung and pancreas. In skeletal muscle an additional weakly expressed transcript of 1.9 kb was detectable. Finally, the smallest transcript of 1.4 kb was the main transcript in heart. This 1.4 kb transcript is also present in skeletal muscle and kidney. By identifying a cDNA clone from heart tissue containing an additional exon with no homology to transketolase sequences, we could isolate part of these alternate transcripts.

Real-time quantification of expression of transketolase family members in healthy tissues

The relative expression level of each member of the transketolase gene family was determined by real-time PCR in the following normal tissues: brain, heart, liver, peripheral blood mononuclear cells, lung, breast, ovary, kidney, testis, spleen, stomach, colon, uterus, esophagus, skin, thymus, bladder, muscle, prostate, and retina. The expression level of the TKT gene in healthy tissues was high compared to the expression of TKTL1 and TKTL2 (not shown). On average the TKT expression level was 60 to 1000-times higher than the TKTL1 or TKTL2 level. The highest level of TKT expression was observed in normal colon, which is 5-10 fold higher compared with the expression level in most other tissues. Under the examination in all other normal tissues, the TKT expression level was very similar with maximal variation of 15-fold differences. In contrast, the expression levels of TKTL1 and TKTL2 were much more tissue-specific and had a greater variation. In testis we observed >12,000-fold higher expression of TKTL1 as compared to lung, ovary, or skin. In testis the TKTL1 expression was even higher than the TKT expression level (twice that of TKT). Strikingly, those healthy tissues (testis, thymus and retina) in which a high aerobic glycolysis has previously been identified were precisely the same tissues in which we identified a high level of TKTL1 expression.
Figure 1: (A) Expression pattern of the human TKTL1 gene on Northern blots of poly(A)+mRNA from different human adult tissues analyzed with a TKTL1 cDNA probe. Four transcripts of 1.4, 1.9, 2.5, and 2.7 kb (arrows) are detectable. Whereas the main transcript in most tissues is 2.5 kb in size, in heart the small transcript of 1.4 is abundant and the 2.5 and 2.7 kb transcripts are missing. Transcript sizes are indicated in kb. (B) Expression of TKTL1 protein isoforms in five tumor cell lines derived from four different tumor entities. Proteins were detected using a MAb specifically detecting TKTL1 protein isoforms and not reacting with other transketolase family members. Each cell line shows a unique expression pattern of TKTL1 protein isoforms. The molecular weight standard is indicated in kDa. (C) The TKTL1 full length protein was expressed in E. coli and was isolated by affinity purification through the N-terminal His-tag. One µg of affinity purified TKTL1 protein was PAGE-separated in a 4-20%-gradient and stained with Coomassie. Proteins of different sizes were detected. The largest protein (66 kDa) represents the N-terminal His-tagged full length TKTL1 protein, whereas smaller TKTL1 proteins are likely due to C-terminal proteolytic cleavage occurring prior to isolation. Note that the migration of the recombinant 66 kDa His-tagged TKTL1-full length protein indicates a size of 75 kDa. Sizes of the protein marker are indicated in kDa.

The monoclonal TKTL1 antibody JFC12T10 detects protein isoforms different in size on Western blot

To determine the TKTL1 expression at the protein level, we raised antibodies against a C-terminal fragment of the recombinant TKTL1 protein in mice. One out of 32 hybridoma cell lines was selected, producing MAb JFC12T10 specifically detecting the recombinant TKTL1 protein in ELISA experiments or on Western blots, and not cross-reacting with recombinant TKT or TKTL2 protein. Using MAb JFC12T10, protein expression of the TKTL1 gene was examined in five different human cancer cell lines representing four different tumor entities. In the lung carcinoma cell line A-549, the breast carcinoma cell line MCF7, the liver carcinoma cell line HepG2, and the colon carcinoma cell lines HCT116 and HT29, different protein isoforms were detected (Figure 1B). The migration of the largest TKTL1 protein isoform in SDS-polyacrylamide gel electrophoresis indicated a molecular size of 75 kDa, which is significantly larger than the size of the open reading frame of the predicted ORF of a TKTL1-cDNA (acc. no. BC25382) encoding a 65.4 kDa protein. This discrepancy was also observed when the His-tagged recombinant 65.4 kDa protein was expressed in E. coli, affinity purified and separated by SDS-PAGE (Figure 1C). The migration of this N-terminal His-tagged 66 kDa TKTL1-full length protein indicated a size of 75 kDa (largest band in Figure 1C), which was the same as the observed size for the native TKTL1 full length protein (Figure 1B). The smaller bands of the recombinant TKTL1 full length protein are likely due to a C-terminal proteolytic cleavage in E. coli prior to the affinity purification by the N-terminal His-tag. As expected from the presence of smaller transcripts in human tissues, smaller protein isoforms were detected in every tested tumor cell line. A weakly expressed protein isoform of app. 58 kDa is present in HCT116 and MCF7 and A549. Even smaller protein isoforms of 40 kDa, 44 kDa and 48 kDa were present in cell line MCF7. Some of these smaller protein isoforms were detectable in the other tested cell lines.
The presence of the five different protein isoforms and the ratio between these protein isoforms was different in all five cell lines.

**TKTL1 is a transketolase**

To determine whether the TKTL1 protein has transketolase activity, recombinant TKTL1 protein expressed in *E. coli* as well as the native TKTL1 protein isolated by affinity purification from a tumor cell line were used. The native as well as the recombinant TKTL1 protein performed a transketolase enzyme reaction using the two substrates xylulose-5-phosphate (X5P) and ribose-5-phosphate (R5P) (Figure 2). As a first step in characterizing the enzymatic properties of TKTL1, the substrate utilization of TKTL1 was tested. Mutation of His103 in yeast transketolase facilitates a one-substrate reaction, thereby demonstrating that this particular amino acid residue determines substrate specificity and reaction modus in yeast transketolase. Since the mutation in TKTL1 led to a deletion of 38 amino acid residues including a His residue homologous to yeast His-103, we determined whether the TKTL1 transketolase is able to perform a one-substrate reaction with X5P as sole substrate. A one-substrate enzymatic activity of 42% in comparison to the two-substrate reaction was observed for the native TKTL1 protein (Figure 2A). A similar one-substrate enzymatic activity (47%) was also observed for the recombinant TKTL1 (Figure 2B). The use of X5P as sole substrate demonstrates a broader substrate utilization and an enhanced one-substrate reaction of the TKTL1 protein. Therefore, similarly to His103 in yeast, the mutation within the TKTL1 protein targeted a protein domain, which modifies substrate utilization and reaction modus of vertebrate transketolases.

In yeast, GAPDH and transketolase were found to be closely bound to each other. We therefore tested whether this protein interaction is also present in humans. To test this, the native affinity purified TKTL1 protein was used for transketolase enzymatic assays without the addition of GAPDH protein. Despite this the observed enzymatic activity (one-substrate or two-substrate reaction) was almost identical to the assay where GAPDH was added (not shown). This demonstrates that during the affinity purification of the native TKTL1 protein a closely bound GAPDH enzyme was isolated. The presence of GAPDH in the affinity purification procedure might have caused the observed higher enzymatic activity in the presence of GAPDH. Therefore, this experiment should be repeated where the GAPDH enzyme is omitted.

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**Figure 2: Determination of transketolase activity of native (A) and recombinant (B) TKTL1 protein.** Two-substrate and one-substrate reaction was determined by the production of NADH as measured by gain in absorbance at 340 nm. Xylulose-5-phosphate (X5P) and ribose-5-phosphate (R5P) were used to determine the two-substrate reaction, whereas X5P alone was used for the one-substrate reaction. One representative of three independent enzymatic assays leading to similar results is shown.
purified TKTL1 protein extract was confirmed by using a combination of TKTL1 and GAPDH antibodies in an ELISA experiment (not shown).

**TKTL1 protein expression**

To determine the role of TKTL1 we performed immunohistochemical analysis to detect the presence of this enzyme in carcinomas (not shown) and healthy tissues. In epithelial cells no expression of TKTL1 protein was observed whereas in a subgroup of epithelial carcinomas a strong TKTL1 expression could be detected. Interestingly, a strong TKTL1 protein expression was correlated to invasive colon and urothelial tumors and to poor patient outcome (Langbein et al., submitted). Endothelial and peripheral neuronal cells are cells which are affected by hyperglycemic conditions in a subgroup of diabetes patients. We therefore determined the TKTL1 expression in endothelial and peripheral neuronal cells of nondiabetic individuals. A strong TKTL1 protein expression was observed in neuronal and endothelial cells (Figure 3). In addition to the expected cytoplasmic staining, a nuclear staining was detected in endothelial cells (Figure 3E,F).

**DISCUSSION**

It has been postulated that the sequencing of the human genome and concomitant advances in genomics and proteomics will have a major impact on the prevention, diagnosis, treatment, monitoring, and outcome of diseases. Application of methods based on genomics and proteomics has enabled us to identify a mutated transketolase-like gene (TKTL1) encoding a full length transketolase protein unless discrimination between the enzymatic activities of the different transketolase enzymes is performed. Furthermore, if an enzyme needs a cofactor, the enzymatic activity can be altered by the cofactor. If more than a single enzyme with an identical cofactor are present, activation of the enzymatic activity by application of a cofactor is the sum of activation of the different enzymes. Transketolase enzyme activities in tumor cells have been activated by application of thiamine, or inhibited by thiamine analogs. The obtained results have been interpreted as the consequence of the TKT gene. We could show that only the TKT1 transketolase is upregulated in tumors, indicating that the TKTL1 transketolase is the relevant transketolase to inhibit.

Known transketolase genes encode a single protein with enzymatic activity, whereas we detected TKTL1 transcripts and proteins different in size. Furthermore, a proportion of the TKTL1 protein(s) is present in the nucleus of tumor cells and healthy cells which is an indication for a multifunctional (moonlighting) protein. The transketolases being characterized so far are homodimers of two full length proteins harboring all typical invariant transketolase amino acid residues. TKTL1 protein isoforms may form TKTL1 homodimers, TKTL1 heterodimers (harboring different TKTL1 protein isoforms), TKTL1/TKT heterodimers and TKTL1/TKTL2 heterodimers. The expression of TKTL1 protein isoforms, even enzymatically non-active, could influence the enzymatic activity of a TKT (or a TKTL2) protein as part of a TKT/TKTL1 (TKTL1/TKTL2) heterodimer. Recently it has been shown that a molecular switch and a proton wire synchronizes the active sites in thiamine-dependent enzymes, indicating that this could also happen in transketolase dimers harboring TKTL1 protein isoforms.

The TKTL1 gene is located in Xq28, one of few chromosomal regions, which are both activated in malignancies and during the cell cycle. The TKT and the TKTL2 gene are not located in such genomic regions. An activation of TKTL1 in the S-phase would not be surprising since there is a strong need for riboses as a basis for new DNA synthesis. The presence of TKTL1 protein within the cytoplasm is in agreement with such an expected enzymatic function. Additionally, nuclei of a subset of neoplastic and non-neoplastic cells do also show a staining. This is very similar to the GAPDH protein. Although the GAPDH protein is a glycolytic enzyme, it executes multiple functions dependent on its localization. Besides diverse membrane and cytoplasmic functions, the nuclear function of GAPDH includes also a role in apoptosis and neurodegenerative disorders.

GAPDH and TKTL1 protein share striking similarities. Both enzymes are evolutionarily ancient proteins involved in sugar metabolism. Both proteins are mainly located in the cytoplasm, but also occur in the nucleus. GAPDH has a function in cell cycle, and TKTL1 is located in a genomic region activated in cell cycle. Both proteins were found to be closely bound to each other in yeast. We also identified TKTL1 and GAPDH as part of a protein complex in humans. The GAPDH protein and
Figure 3: Expression of TKTL1 in peripheral neuronal cells and endothelial cells of nondiabetic individuals. (A-D) Strong cytoplasmic expression in peripheral neuronal cells, no expression in surrounding stroma cells. (E-F) Strong cytoplasmic and nuclear expression in endothelial cells.
transketolase proteins/enzyme reactions have been associated with neurodegenerative diseases. Furthermore, an orally active anti-apoptotic molecule (CGP 3466B) that binds to GAPDH has been used as a potential treatment for neurodegenerative disease.

In diabetic patients a subnormal erythrocyte transketolase enzyme activity has been identified. Hyperglycemic conditions cause the development of chronic diabetic complications including retinopathy and loss of vision, nephropathy and end-stage renal disease, macro- and microvascular damage, and peripheral neuropathy. Vascular endothelial cells and other cell types damaged by hyperglycemia are uniquely unable to downregulate glucose transport when exposed to extracellular hyperglycemia. Three major biochemical pathways implicated in the pathogenesis of hyperglycemia-induced vascular damage have been identified: the hexosamine pathway, the advanced glycation end product (AGE) formation pathway, and the diacylglycerol (DAG)-protein kinase C (PKC) pathway. Activation of transketolase enzyme reactions by the lipid-soluble thiamine derivative benfotiamine inhibits these three pathways as well as hyperglycemia-associated NF-κB activation. Therefore activation of transketolase enzyme reactions represents a successful therapeutic intervention to prevent chronic diabetic complications.

Besides this, transketolase enzyme reactions represent also a target for therapeutic intervention in cancer. Transketolase enzyme reactions of the nonoxidative PPP play a crucial role in nucleic acid ribose synthesis utilizing glucose carbons in tumor cells. More than 85% of ribose recovered from nucleic acids of certain tumor cells derives directly or indirectly from the nonoxidative part of the PPP. The importance of transketolases for tumor cell metabolism is underlined by the fact that the application of specific transketolase inhibitors to tumors induces a dramatic reduction in tumor cell proliferation. In addition, the activation of transketolases by application of thiamine stimulates tumor growth.

To evaluate the role of the three transketolase genes in diseases, we analyzed in a first step the expression of all three members of the TKT gene family in cancer. We could demonstrate that TKTL1 is specifically upregulated in malignancies at the mRNA level, whereas TKT and TKTL2 are not upregulated. We confirmed the over-expression of TKTL1 transketolase on the protein level. The clinical importance of TKTL1 upregulation could already be demonstrated, since TKTL1 expression was correlated to invasive colonic and urothelial tumors and to poor patient outcome (Langbein et al., submitted). These findings suggest TKTL1 as the relevant target for novel anti-transketolase cancer therapies.

Transketolase enzyme reactions represent the rate-limiting step of the nonoxidative part of the PPP. Upregulation of the TKTL1 transketolase allows an enhanced nonoxidative glucose degradation via the PPP. An enhanced glucose metabolism in tumors, which is characterized by the anaerobic degradation of glucose even in the presence of oxygen (aerobic glycolysis) leading to large amounts of lactate was already identified by Otto Warburg 80 years ago (known as the Warburg effect). The relevance of the Warburg effect to cancer cell biology is still discussed controversially. Recently, this type of glucose metabolism has gained increased attention as an important step in carcinogenesis. The widespread clinical use of positron emission tomography (PET) for the detection of enhanced glucose metabolism in invasive tumors and metastases has in particular rekindled Warburg’s theory. In addition, the PET technology is also being clinically applied for the detection of a reduced glucose metabolism. In AD patients a reduced cerebral glucose metabolism can be visualized in several cortical regions, even before the onset of clinical symptoms.

In neurodegenerative diseases, diabetes and cancer an altered glucose metabolism as well as altered transketolase proteins/enzymes have been detected. There is strong evidence that there is a common molecular and biochemical basis for these diseases. We postulate a novel metabolic pathway, which links tumor metabolism, chronic diabetic complications, vascular damage, aging and neurodegenerative diseases. The proposed pathway is based on the mutated transketolase TKTL1 and its novel enzymatic properties (Figure 4B).

To understand the role of TKTL1 for health and disease, it is necessary to put the information about the TKTL1 gene/protein in the context of what has been known about transketolase enzyme reactions, the three-dimensional structures of transketolases, known transketolase mutants and biochemical pathways in which transketolases are involved. The nonoxidative part of the PPP presented in textbooks (Figure 4A) is based on an interpretation of the results of catalysis by liver and pea enzyme preparations incubated with variously labeled 14C-ribose phosphate substrates and the resultant formation of 14C-labeled fructose-6-phosphate. Since the PPP reactions can be part of recycling reactions between different sugars, labeled C-atoms are randomized. The initial choice of acetone powder enzyme systems led to a blockade of any oxidation of hexose carbon, prevented the subsequent randomization of the 14C isotope in hexose-6-phosphate, and permitted the investigation of the 14C-labeling pattern in the hexose-6-phosphate products after only one passage of ribose-5-phosphate carbon through the reactions of the PPP. These reactions were tentatively attributed to catalysis by transketolase and transaldolase. The conclusion concerning the nature and order of these reactions is still controversial because the degree of 14C isotope labelling and its distribution in carbon atoms of fructose-6-phosphate differed from that predicted by reaction sequences. Wood and Katz mathematically developed the idea of reshuffling and recycling to explain the observed labelling results. The observed 14C labeling results in an experimental system with a blocked randomization of C-atoms are in contrast to the PPP reactions in textbooks. As this fundamental pathway is used as a basis for the interpretation of important bio-
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chemical results related to transketolase enzyme reactions, glucose metabolism, nucleotide synthesis, and energy production, the PPP reactions presented in textbooks should be re-evaluated.

It is remarkable that the initial events in pre World War II years leading to the unraveling of the PPP were powerfully influenced by the force of Nobel laureate Otto Warburg’s conviction that NADPH was the source of reducing equivalents for respiration. The search for an enzyme activity was successful, and NADPH-cytochrome c reductase (EC 1.6.2.4) was isolated from yeast and liver. However, by that time there was growing evidence that the oxidation of NADPH was not a significant source of reducing equivalents for respiration and the entire proposal was put to rest in 1951 when Lehninger demonstrated that NADH was the substrate for oxidative phosphorylation in respiring mitochondria. Therefore the NADPH generated in the oxidative part of the PPP could be used for oxidative phosphorylation in respiring mitochondria and may be underestimated in cells with an upregulation of TKTL1. Tumor cells or healthy cells with an up-regulation of TKTL1 could use the generated NADPH as reducing equivalents as well as for oxidative phosphorylation.

TKTL1 is a transketolase with characteristics never yet observed in a transketolase. During the evolution of higher vertebrates mutations within one of three transketolase genes led to a mutated, but functionally active transketolase enzyme as well as smaller protein isoforms. Yet all characterized transketolases have similar kinetic and physical properties, but the mammalian enzymes are more selective in substrate utilization than the nonmammalian. The most prominent difference between the TKTL1 protein and known transketolase proteins is the deletion of an internal stretch of amino acid residues homologous to yeast transketolase amino acid residues 68–107). Indeed, the TKT and TKTL1 proteins are well conserved with yeast transketolase, whose crystallographic structure has been determined and for which extensive enzymatic analysis has been performed. Structural analysis of yeast transketolase has identified amino acid residues (HIS30, HIS263, ARG359, SER386, HIS469, ASP477, ARG528) which build the substrate channel.

X5P, was increased more than tenfold in two mutants. Besides the alterations in substrate specificity the mutation also changed the reaction modus. Mutation of His103 in yeast transketolase led to a significant acceleration of a one-substrate reaction and a slow down of the two-substrate reaction so the rates of both types of catalysis becoming equal. These authors also demonstrated that the type of substrate reaction (one-substrate or two-substrate) and the affinity to thiamine is influenced in the His103 yeast transketolase mutant protein dependent on the presence of Ca(2+) or Mg(2+). Taken together, these results indicate that the TKTL1 protein, lacking the invariant His103, may also have an altered substrate spectrum, reaction modus, and thiamine affinity and may allow a regulation by Ca(2+) or Mg(2+). The enhanced TKTL1 one-substrate reaction, which we observed in our enzymatic assays, indicates that the deletion within the TKTL1 protein has an important influence on the enzymatic properties of vertebrate transketolases.

Bjyova et al. described a one-substrate reaction for the wild type yeast transketolase. Apart from the common two-substrate reaction with ketose as donor substrate and aldose as acceptor substrate, the yeast transketolase enzyme splits X5P into glyceraldehyde-3-phosphate and erythulose. Erythulose is formed by the action of free and enzyme-linked glycolaldehyde. Using the recombinant as well as the human TKTL1 protein our results confirm the formation of glyceraldehyde-3-phosphate in a one-substrate reaction utilizing X5P as the sole carbon source. An enzyme specialized to perform such a one-substrate reaction with X5P is present in heterofermentative lactic acid bacteria. This phosphoketolase (E.C. 4.1.2.9) belongs to the family of thiamine-dependent enzymes and splits X5P to glyceraldehyde-3-phosphate and acetylphosphate, thereby enabling a metabolic pathway leading to lactate, and acetate or ethanol. An intermediate product of this metabolic pathway is acetyl-CoA. If the metabolism of X5P via TKTL1 results in the formation of acetyl-CoA too, this would allow an energetically even better pathway for fermentative glucose degradation than the PPP already does. If this is the case, the mutation in the TKTL1 protein represents an example of evolutionary convergence, leading to a ketolase enzyme reaction already evolved earlier in the history of life.

The upregulation of TKTL1 transketolase has been correlated to invasive colonic and urothelial tumors (Langbein et al., submitted). The transketolase enzyme reactions and other reactions of the PPP allow glucose conversion to ribose for DNA and RNA synthesis and generation of NADPH as a reducing agent for biosynthesis. These are necessities for a growing tumor cell. In addition, the nonoxidative part of the PPP controlled by transketolase enzyme reactions, allows an anaerobic glucose degradation. Anaerobic conditions are often present in tumors and are limiting the growth of tumors. Even in pre-malignant lesions often characterized as highly vascularized, near-zero partial oxygen pressures are observed at distances of only 100 µm from...
vessels. Tumor cells with an upregulation of transketolase enzyme reactions enhance the nonoxidative glucose usage and therefore are adapted for a growth without oxygen. Anaerobic glucose metabolism leads to the production of lactate, which allows matrix degradation and tumor invasiveness. Both glucose usage as well as lactate production have been identified as prognostic markers indicating poor prognosis of cancer patients. The important role of transketolase enzyme reactions for lactate production has already been determined and a lactate-based matrix degradation has been described by Stern et al. However, the transketolase enzyme responsible for this has not been identified yet. Our findings demonstrate that a single transketolase (TKTL1) is upregulated in tumors, whereas other known transketolases are not upregulated. Since transketolase enzyme reactions control the nonoxidative part of the PPP, upregulation of any of the three transketolases should lead to an enhanced anaerobic glucose use. Although the nonoxidative PPP allows an anaerobic glucose degradation, energetically it is as inefficient as the anaerobic glucose degradation via the Embden-Meyerhof pathway. Glucose degradation via the Embden-Meyerhof pathway and the PPP leads to lactate if oxygen is absent. Muscle tissue switches to the final-oxidation metabolism if oxygen is present (Pasteur effect). Tumor tissues do not (Warburg effect). Why do tumor cells still continue to degrade glucose to lactate even in the presence of oxygen if this anaerobic pathway is energetically inefficient? Why do healthy tissues like retina and testis, in which TKTL1 is strongly expressed, show aerobic glycolysis? The energetic output of anaerobic glucose degradation, either based on the Embden-
MUTATIONS IN THE TRANSKETOLASE-LIKE GENE TKTL1

Figure 4B

Meyerhof pathway or the PPP reactions presented in textbooks, is worse compared to the oxidative glucose degradation. An analysis of the contribution of different fuels and metabolic pathways in proliferating MCF-7 breast cancer cells has demonstrated that 65% of the total ATP turnover is from unidentified sources. An explanation for the observed tumor metabolism (aerobic glycolysis/anaerobic glucose degradation) and the unidentified ATP source in tumor cells could be the TKTL1 transketolase, if the mutation leads to a (phos-pho)ketolase enzyme.

The new proposed TKTL1 (phospho)ketolase reaction and the concomitant link between pentose PPP and Embden-Meierhof pathway could be the underlying cause of the metabolism of invasive tumors as well as a number of healthy cell types and tissues. In tumor cells, activation of this metabolism could lead to a selective growth advantage. In healthy cells (e.g. neuronal cells, endothelial cells, retinal cells) TKTL1 enzyme activity could reduce an elevated glucose level and prevent or reduce AGE formation and generation of radicals. The proposed model would confirm findings and postulates of the Nobel laureates Otto Warburg and Albert Szent-Györgyi. Otto Warburg described an anaerobic glucose metabolism of tumors, even in the presence of oxygen. Albert Szent-Györgyi divided life into a first anaerobic period, the so-called alpha period (alpha state), followed by a second aerobic period (beta period), in which oxygen is the universal electron acceptor. He assumed that before light and O2 appeared, a weak electron acceptor could have occurred through linkage of two C=O groups to glyoxal and addition of a methyl group. In addition he and his coworkers identified the reaction of aldehydes and ketones with proteins leading to Schiff bases. Those reactions contribute to the chronic complications observed in diabetes patients. Furthermore he postulated that cancer cells are trapped in the alpha state (anaerobic period of life). A TKTL1 based anaerobic pathway concomitant with an electron transfer to methylglyoxal would indeed enable an oxygen independent electron transport.
The postulated TKTL1-based metabolism is in agreement with observed changes in the metabolism of diabetic patients and tumors. The cleavage of X5P to GAP and a C2-unit (likely acetyl-CoA) might explain the protective effect of the TKTL1 activity in hyperglycemic conditions (diabetic patients, high carbohydrate diets). The aldehydes and ketones would be converted to a non-toxic C2-unit, which might also contribute to the synthesis of protective lipids. Both effects would prevent or reduce the formation of AGE and radicals, thereby preventing chronic hyperglycemic damage and neurodegeneration. Tumor cells would also obtain a selective growth advantage by the up-regulation of TKL1. It is well known that a high acetyl-CoA concentration ultimately blocks the pyruvate-decarboxylase reaction leading to accumulation of pyruvate and L-lactate (observed in aggressive cancer cells46) and thereby confer a selective growth advantage to invasive and matrix degrading tumor cells. A high acetyl-CoA concentration will also provide the basic energy. A high concentration of GAP will lead to an enhanced reaction sequence of the lower Embden-Meyerhof pathway reactions. Despite the accumulation of C3-metabolites down to phosphoenolpyruvate (PEP), only very low levels of pyruvate are formed this way, because an almost inactive pyruvate kinase isoform (tumor M2PK) is upregulated in tumors44. Both the fatty acid synthase55 as well as the tumor M2PK56 represent prognostic markers for a poor prognosis for cancer patients.

Furthermore, an important bypath of glycolysis has attracted little attention by biochemical textbook writers. DHAP can enzymatically be metabolized to methylglyoxal und further to D-lactate and pyruvate (see Figure 4). Since the biosynthesis of methylglyoxal is dependent on high DHAP concentrations, downregulation of the glycerol-3-P dehydrogenase, another enzyme utilizing DHAP, is a prerequisite. As shown by Howard et al.57, late stage cancer cells no longer synthesize triacylglycerides from glycerol-3-P because of complete depletion of this metabolite. Besides its role for the generation of chronic diabetic complications, methylglyoxal is a potent cytotoxin and a very reactive aldehyde known as a key metabolite in blocking cell proliferation58. It has already been suggested that enhanced degradation of methylglyoxal via upregulated glutathione-dependent glyoxylase reactions are key steps for aggressive tumor cells, ultimately leading to cell proliferation signals59. The inhibition of glyoxylase represents a target of current anti-tumor strategies. The inhibition of glyoxylase I by methotrexate has been shown to lead to anti-tumor activity in children with acute lymphoid leukemia activity through the accumulation of methylglyoxal60.

We postulate a TKTL1-based biochemical pathway which explains:

a) the tumor metabolism of anaerobic glucose degradation leading to lactate (Warburg effect/aerobic glycolysis) and the matrix degradation/invasiveness of tumors
b) the glucose metabolism in healthy tissues (e.g. testis, retina) showing anaerobic glucose degradation (aerobic glycolysis)
c) the onset of chronic complications in diabetes patients
d) the higher cancer mortality of diabetes patients
e) a sugar/AGE-dependent development of neurodegenerative diseases
f) a sugar/AGE-dependent aging process

The basis of this proposed biochemical pathway is the mutated transketolase TKTL1 (Figure 4). We predict that the deletion of a former coding exon leads to a (phospho)ketolase similar to the phosphoketolase present in lactobacillae. In addition to the conventional two substrate reaction, the TKTL1 (phospho)ketolase could perform a one-substrate reaction using X5P as sole substrate. Using this enzyme an anaerobic glucose degradation would be possible, which would generate more energy than the lactate fermentation via the Embden-Meyerhof pathway. The enzymatic reaction of TKTL1 would cleave X5P to GAP and a C2-unit. Enhancing the TKTL1 ketolase activity would reduce the glucose level and would prevent enhanced AGE formation. The activation of TKTL1 enzyme activity could lead to a reduction of AGE formation. TKTL1 variants with a higher K_m (reduced activity) could predispose to enhanced AGE formation, accelerated aging and accelerated neurodegeneration.

Neoplastic cells would strongly benefit from an up-regulation of TKTL1 as this would lead to a selective growth advantage. Clinical studies have already confirmed this important role of TKTL1 for cancerogenesis as TKTL1 upregulation could be correlated to invasive colonic and urothelial cancers and patients with poor outcome (Langbein et al., submitted). Tumors with an upregulation of TKTL1 could use huge amounts of glucose and produce large amounts of lactate. Those tumors would benefit from an elevated blood glucose level. This could explain why diabetes is an independent predictor of mortality from cancer of the colon, pancreas, female breast, and, in men, of the liver and bladder61 and why patients with diabetes mellitus and high-risk stage II and stage III colon cancer experience a significantly higher rate of overall mortality and cancer recurrence62. Database searches have indicated the presence of a TKTL1 orthologue in higher vertebrates like the mouse and rat, but the absence of a TKTL1 orthologue in lower vertebrates such as frog, fish and shark. Future analysis will show if lower vertebrates indeed have no TKTL1 orthologous enzyme and if invasive tumors with aerobic glycolysis are absent.
If the TKTL1-based glucose metabolism is important for tumorigenesis, the absence of TKTL1 enzyme activity should inhibit tumor proliferation. Inhibition of TKTL1 enzyme activity can be performed by cofactor analogues, substrate analogues, inhibitory small compounds and depletion of substrates. Inhibition of transketolase enzyme reaction by cofactor analogues (e.g. oxythiamine) and small compounds (Genistein, AVE-MAR) have already been performed, and tumor proliferation could be successfully inhibited. Future analysis will show whether the tumor-inhibitory effect is due to an inhibition of TKTL1 transketolase. Since a depletion of glucose would lead to an inhibition of TKTL1, a diet would also have an anti-tumor effect if the TKTL1-mediated pathway is important for tumor growth. A diet with low glucose content or depleted of glucose and rich in short fatty acids has been suggested as a nutraceutical for the inhibition of tumor growth (KlöR, pers. comm.), and a ketogenic diet has already been successfully applied to cancer patients. Interestingly, a similar diet has been suggested to prevent AGE formation and neurodegenerative diseases. It has been postulated that the primary event leading to the development of AD is consumption of an evolutionarily discordant high carbohydrate diet. We postulate that the mutations in the TKTL1 gene lead to a (phospho)ketolase enzyme as the basis of a novel biochemical pathway in higher vertebrates, which represents a missing link for the understanding, prevention and therapy of macro- and microvascular damage, neuropathies, neurodegenerative diseases and invasive cancer.

Experimental Procedures

Real-time PCR
RNA was extracted from tumor and corresponding normal tissue using a commercial kit (Qiagen). For cDNA synthesis, 1 µg total RNA was DNase I digested for 15 min at 25 °C in a final volume of 20 µl containing 1 µl DNase I Amp Grade (1 Unit/µl; Invitrogen) and 2 µl DNase reaction buffer (10x; Invitrogen). The reaction was stopped by adding 2 µl EDTA (25 mM; Invitrogen) and incubated for 10 min at 65 °C. Reverse transcription was performed for 2 h at 37 °C in a final volume of 40 µl containing 4 µl 10x RT buffer, 4 µl 5 mM dNTP, 1 µl RNAsin 40 U/µl (Promega), 4 µl Oligo 15 µg/ml and 2 µl Omniscript 4 U/µl (Qiagen). Reverse transcriptase was inactivated by heating at 93 °C for 5 min and cooling at 4 °C for 5 min. PCR was performed with SYBR Green I dye using the QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer’s protocol. The PCR reactions were carried out in a total volume of 25 µl and with a primer concentration of 200 nM. 6.25 ng cDNA of oligo dT primed total RNA was added to each real-time PCR. Real-time PCR was performed using the following parameters: 50 °C for 2 min, denaturing at 95 °C for 10 min, 40 cycles of 95 °C at 15 s and 1 min at 68 °C. The β-actin gene was used as an endogenous control and each assay included standard samples in duplicates. Data analysis was carried out using PerkinElmer Biosystems analysis software and according to the manufacturer’s manuals. To control the specificity of the amplification products, a melting curve analysis was performed (ABI Prism Dissociation Curve Software, PE Applied Biosystems). In addition, PCR products were gel separated to confirm the bands of the expected size. Finally, results, expressed as N-fold differences in target gene expression relative to the reference gene β-actin, termed ‘Ntarget’, were determined as follows:

\[ \text{N}_{\text{target}} = 2^{(\Delta \Delta \text{Ct})} \]

where delta Ct values of the sample and reference are determined by subtracting the average Ct value of the test gene from the average Ct value of the β-actin gene. The following primers (sequences 5’ to 3’) were used for the amplification of the transcripts of the three known human transketolase genes, TKTL1 (TAACCATGACGCCTACTG; CATCCTAAAGCCTTTGCTG), TKT (TGTGTCAGTGACGATGTTG; ACCATTCATCCCCTTAG), TKTL2 (AAACATGGCTTATTCTAAAAAGTCAGG; GCCTTGCTTTAAAAAGACGAG).

The β-actin gene (CCTAAAGGCCACCCCCCTCTC; ATGCTACCCCTCCCCGTGTTG) was used as a control. Primers for TKT, TKTL1, TKTL2 and β-actin gene were designed using PRIMER (Husar program package, DKFZ Heidelberg) and Primer Express (PE Applied Biosystems) software.

Tumor Cell Lines
The lung carcinoma cell line A549, the breast carcinoma cell line MCF7, the liver carcinoma cell line HepG2, and the colon carcinoma cell lines HCT116 and HT29 were obtained from ATCC. Cells were grown in RPMI 1640 or DMEM supplemented with 10% FCS, penicillin and streptomycin (Invitrogen) at 37 °C with 5% CO2.

Northern blot analysis
A DNA probe from the 3’ untranslated region (residue 1627 to 2368) of the TKTL1 transcript (acc. no. X91817) was labeled with [(alpha)-32P]dATP and [(alpha)-32P]dCTP (3000 Ci/mmol) in a random primed reaction. A multiple human adult tissue poly(A)+ RNA Northern blot was purchased from BD Biosciences Clontech. Hybridization was carried out in 0.5 M sodium phosphate, 7% SDS, 0.2% bovine serum albumin, 0.2% PEG 6000, 0.05% polyvinylpyrrolidone 360000, 0.05% Ficoll 70000 and 0.5% dextran sulfate at 65 °C overnight. Non-specifically bound probe was removed by washing at 65 °C in 40 mM sodium phosphate, pH 7.2, 1% SDS for 60 min. Filters were exposed to X-ray film (Kodak) for 1-5 days.
Western blot analysis

For Western blot analysis of native TKTL1, cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM sodium chloride, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.02% sodium azide, 1 mM phenylmethylsulfonyl fluoride). Aliquots of 50 µg soluble protein were loaded into each well, electrophoresed on 12.5% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membranes (Millipore). For detection of TKTL1-proteins the HRP-coupled JFC12T10 MAb was used in a final concentration of 1 µg/ml. The MAb was visualized with an ECL Western blot detection system (Amersham Pharmacia Biotech). For Western blot analysis of recombinant TKTL1 a PAGE separation on a 4-20% gradient gel was used.

Expression of recombinant TKTL1 protein

The TKTL1 open reading frame of cDNA sequence (acc. no. BC025382) was cloned into the pDEST17 vector (Invitrogen) and transformed into the E. coli strain BL21-AI (Invitrogen). Expression was induced with 0.2% arabinose at 21 °C for 4 h. Crude cell lysate was prepared in lysis buffer (20mM Tris[ph7.5], 5mM imidazole, 5 mM β-mercaptoethanol, 500 mM NaCl, and 1% Triton X-100) by freezing (dry ice, 10 min) and thawing (37 °C, 5 min) 3 times. Soluble protein fractions were obtained by centrifugation of the cell lysate at 12,000 g for 30 min at 4 °C. His6-TKTL1 protein was purified with NiNTA resin (Qiagen) according to the manufacturer’s instructions with elution buffer containing 200 mM imidazole. Imidazole and salt were subsequently removed by dialysis against 0.1 M Tris (pH 7.5). The purified enzyme was stored at –20 °C in 40% glycerol and 0.1% dithiothreitol (DTT).

Affinity-purification of native TKTL1 protein from human chronic myelogenous leukemia K562 cell line

10 mg of MAb JFC12T10 was coupled to 2 ml carbo-link according to the manufacturer’s instructions (carbo-link; Pierce). K562 cells were grown in serum-free media (ISF-1, Invitrogen BioTech Services GmbH). After centrifugation, the pellet of 2.2x10⁹ cells was resoluted in 50 ml PBS containing protease inhibitor cocktail (Roche). Cell lysis was performed using a French press, followed by a centrifugation at 50,000 g. The supernatant was filtered (0.2 µm) and binding of supernatant to affinity material was performed overnight at 4 °C (batch mode). After transfer to a column, a wash procedure was performed with 150 mM PBS buffer pH 7.4. For elution of column-attached proteins 100mM glycine-HCl pH 2.0 was used. Two protein peaks, detected using a UV 280 nm-based detection system, were collected and neutralized with Tris pH 7.4.

Measurement of enzymatic activity

The activity of TKTL1 was measured by a coupled enzyme assay at 25 °C. Reactions were started by addition of recombinant or native TKTL1 protein and determined spectrophotometrically by the rate of reduction of NAD⁺ in the following reaction sequence: xylulose-5-phosphate (X5P) and ribose-5-phosphate (R5P) > (TKTL1 activity) > glyceraldheyde-3-phosphate and sedoheptulose-7-phosphate > (glyceraldehyde-3-phosphate dehydrogenase activity [GAPDH]) > NAD⁺ → NADH + H⁺, 1,3-phosphoglycerate. Transketolase two-substrate activity was determined in the following reaction (final concentrations): 4 mM X5P, 4 mM R5P, 500 µM NAD⁺, 2 mM MgCl₂, 0.9 mM PBS, 0.2 mM thiamine PM, 5 µg recombinant TKTL1 protein, or 4 µg native TKTL1 protein, 3 U GAPDH, 0.15 mol/l Tris buffer pH 7.4 in a reaction volume of 1 ml. Transketolase one-substrate activity was determined by omitting R5P, using X5P solely as substrate. GAPDH was obtained from Sigma.

Hybridoma generation

Anti-TKTL1 MAb producing cells lines were selected following immunization of BalbC mice with a human recombinant 22 kDa C-terminal TKTL1 protein fragment. Spleen cells from four immunized mice and mouse myeloma Ag8 cells (ATCC) were mixed and fused with PEG1500 (Roche). The suspension was transferred into RPMI 1640 media (Sigma), containing 20% FBS (Gibco), 10% hybridoma cloning factor (Biochrom), 1x hypoxanthine-aminopterin-thymidine (Sigma), 0.1% gentamycin (Gibco) and distributed in 20 flat-bottom microplates. Two to 3 weeks after fusion, supernatants from wells with positive growth were collected. MAb binding to the recombinant 22 kDa C-terminal TKTL1-fragment as well as to an unrelated recombinant control (E. coli cytosine deaminase) was tested by ELISA. Cells producing MAbs with binding to recombinant 22kDa C-terminal TKTL1-fragment and not reacting with the unrelated control protein were expanded in hypoxanthine-thymidine media and subjected to two cycles of recloning at limiting dilution (1 cell/well). Clones showing strong ELISA reactivity toward TKTL1 recombinant protein and no crossreaction against cytosine deaminase were further characterized for reactivity with TKTL1 recombinant protein and crossreactivity with TKT and TKTL2 recombinant proteins on Western blot. Clones with reactivity against TKTL1 and no crossreactivity against TKT or TKTL2 protein on Western blot were tested on paraffin-embedded sections of human gastric carcinoma with and without an over-expression of the TKTL1 gene on the mRNA level. MAb-containing supernatants were produced by culturing hybridomas in normal tissue culture media followed by an adaptation to serum and protein-free media. MAbs were affinity-purified by use of genetically engineered protein-G (Invivo BioTech Services GmbH).
Tissue samples and immunohistochemical staining

Surgical resection specimens were obtained from patients undergoing surgery for colon, gastric or bladder cancer at the Department of Surgery, University Hospital Mannheim, Faculty of Clinical Medicine of Ruprecht-Karls-University Heidelberg, Germany and from Clinomics Biosciences. 3 μm thick paraffin tissue sections from non-diabetic individuals were heated in a micro-wave oven for antigen unmasking in 10 mM sodium citrate (pH 6.0) for 5 minutes at 900 W, for 5 min at 900 W in dH2O, and for 5 min in 10 mM sodium citrate (pH 6.0) at 900 W. After a wash in phosphate/saline buffer (PBS), inhibition of endogenous peroxidase was per-formed as described above. Sections were then exposed for 15 min to biotin-avidin blocking buffer (Vector Laboratories). Blocking of non-specific staining was performed with goat serum as described above. Primary antibodies were visualized with avidin-biotinylated horseradish peroxidase complex (ABC) and diaminobenzidine tetra-hydrochloride (DAB) (Elite kit; Vector Laboratories), and counterstained with Mayer’s hematoxylin.

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