Expression of the mutated transketolase TKTL1, a molecular marker in gastric cancer

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Abstract. The nonoxidative pentose phosphate pathway allows glucose conversion to ribose for DNA or RNA synthesis and glucose degradation to lactate controlled by transketolase enzyme reactions. It has been postulated, that this pathway is of the utmost importance in tumors for the proliferation process. We detected a strong upregulation of the mutated transketolase transcript (TKTL1) in a considerable number of patients with gastric cancer (GC) or cancer of the gastroesophageal junction (GEJ). While only 10.8% of the cancer tissues revealed a significant mRNA upregulation, 36.9% of the cancer tissues demonstrated a protein overexpression. We propose that TKTL1 upregulation is a common phenomenon in GC and cancer of the GEJ leading to an enhanced, oxygen-independent glucose usage which might contribute to a more aggressive tumor growth. Since molecular targeted inhibition of transketolase enzyme reactions suppresses tumor growth and metastasis, TKTL1 could be a relevant target for anti-transketolase therapies in gastric cancer.

Introduction

Gastric cancer is still the second most common cancer worldwide, however, with considerable incidence variation across different countries. In Western Europe the incidence of gastric cancer has been declining steadily over the last decades, but still contributes significantly to cancer mortality (1,2). Despite technical advances and the use of adjuvant therapy, the substantial mortality associated with gastric cancer prevails. Patients are at high risk for early lymph-node metastases and local recurrence with very poor survival. However, in the case of early gastric cancer, advanced endoscopic techniques allow local minimalized therapies [e.g. endoscopic mucosal resection (3) or endoscopic full-thickness resection (4)]. The application of these techniques must be restricted to well or moderately differentiated tumors solely invading the mucosa and with low risk of lymph node metastasis (5,6). Clinical problems often arise in the pre-therapeutic risk assessment of these tumors for an adequate therapeutic strategy. Resulting from these clinical problems, tumor markers characterizing the malignant potential or elucidating pathways with potential for therapeutic interventions are of interest.

Though alterations in the nonoxidative pentose phosphate pathway (PPP) are considered to be very important in tumor proliferation processes (7), there is little evidence on their importance in gastric cancer. In this pathway the thiamine (vitamin B1) dependent transketolase enzyme reaction plays a crucial role finally leading to nucleic acid ribose synthesis through utilizing glucose carbons. More than 85% of ribose recovered from the nucleic acids of certain tumor cells derives directly or indirectly from the nonoxidative part of the PPP controlled by transketolase enzyme reactions (8). Experimentally, the application of specific transketolase inhibitors leads to a reduced tumor cell proliferation (9). In addition, activation of transketolase by the application of thiamine stimulates tumor growth (10).

However, the molecular basis of this phenomenon in the carcinogenesis of solid tumors is poorly understood. Transketolase reactions in the nonoxidative PPP are complex because of the presence of three human transketolase genes: TKT, TKTL1 and TKTL2. TKT is known to encode an active transketolase enzyme (11) similar to TKTL2. TKTL1 is assumed to be a pseudogene. However, we have demonstrated that TKTL1 in fact encodes a transketolase enzyme (12).

Using the real-time PCR technique we recently described a strong upregulation of TKTL1 in several solid carcinomas whereas the TKT and TKTL2 transcripts were not overexpressed. This led to the identification of TKTL1 as a paralogue of the known transketolase genes with tumor specific overexpression of its transcript and the encoded protein (13).

The present study evaluates the role of TKTL1 in gastric cancer (GC) and cancer of the gastroesophageal junction

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(GEJ). Of major interest was the TKTL1 expression in tumor-specific transketolase metabolism and its potential as a tumor and differentiation marker. To determine the value of TKTL1 expression levels, mRNA expression was examined using the real-time PCR technique and protein expression with immunohistochemical methods. Additionally, the clinicopathological implications of the TKTL1 expression levels were studied.

Patients and methods

Patients and tissue samples. The specimens of 65 consecutive patients with histologically verified primary GC or cancer of the GEJ who underwent surgery between December 1999 and June 2002 at the Department of Surgery, University Hospital Mannheim, were examined. The study procedures were approved by the local ethics committee. Informed consent was obtained from each patient. Cancerous tissue and matched normal gastric mucosa was available from all the patients. No patient had received prior (radio-) chemotherapy. For RNA extraction, the specimens obtained during the operation were immediately snap-frozen in liquid nitrogen and stored at -80˚C until further processing. For immunohistochemistry the specimens were fixed in 4% buffered formalin for 24 h and paraffin-embedded. The age, sex, tumor differentiation, Lauréns classification, tumor location and pTNM stage (5th edition, 1997) were recorded and categorized by reviewing the medical charts and pathological records.

Real-time PCR. The details have been described previously (12).

Antibody. Mouse monoclonal IgG antibody against TKTL1 (clone JFC12T10; mouse IgG2b) was used (R-Biopharm AG, Darmstadt, Germany; Linaris GmbH, Wertheim-Bettingen, Germany).

Immunohistochemistry. Formalin-fixed and paraffin-embedded sections (3 μm) of matched normal and cancerous mucosa tissue were dried at 37˚C overnight, dewaxed using xylene, rehydrated in a series of graded alcohols and rinsed with distilled water. The first section was stained with H&E for histological review. Additional sections served for immunohistochemistry. TKTL1 reaction was carried out as follows: Antigen retrieval was performed in 10 mM sodium citrate (pH 6.0) in a microwave oven 3x for 5 min at 900 W. After washing in phosphate saline buffer (PBS), inhibition of endogenous peroxidase was performed by a 5 min incubation with 3% H_2O_2/methanol. The sections were then exposed for 15 min to a biotin-avidin blocking buffer (Vector Laboratories, Burlingame, CA, USA). After two washes in Tris-saline buffer (TBS), the slides were incubated with 1% goat serum for 30 min and subsequently exposed to mouse anti-TKTL1 (clone JFC12T10; mouse IgG2b) antibody (25 μg/ml) overnight at 4˚C. A secondary biotinylated antibody and a horseradish conjugated streptavidin complex (Elite kit; Vector Laboratories) followed by incubation with 3'-diaminobenzidine was used to visualize the primary antibody binding site according to the manufacturer's instructions. Appropriate positive and negative controls were included in each reaction.

Table I. Clinical features of the 65 patients included in this study.

Characteristic	GC[a] (%) n=42 (64.6)	GEJ[b] (%) n=23 (35.4)	Both (%) n=65
Age			
median	67	63	66
(range)	(38-87)	(35-83)	(35-87)
Gender			
male	25 (59.5)	17 (73.9)	42 (64.6)
female	17 (40.5)	6 (26.1)	23 (35.4)
pT			
1	3 (7.1)	2 (8.7)	5 (7.7)
2	19 (45.2)	10 (43.5)	29 (44.6)
3	16 (38.1)	9 (39.1)	25 (38.5)
4	4 (9.5)	2 (8.7)	6 (9.2)
pN			
0	13 (31)	7 (30.4)	20 (30.8)
1	11 (26.2)	12 (52.2)	23 (35.4)
2	12 (28.6)	1 (4.3)	13 (20)
3	6 (14.3)	3 (13)	9 (13.8)
pM			
0	37 (88.1)	21 (91.3)	58 (89.2)
1	5 (12)	2 (8.7)	7 (10.8)
Grading			
2	13 (31)	10 (43.5)	23 (35.4)
3	29 (69)	13 (56.5)	42 (64.6)
UICC-stage			
IA	2 (4.8)	2 (8.7)	4 (6.2)
IB	11 (26.2)	3 (13)	14 (21.5)
II	5 (12)	6 (21.6)	11 (16.9)
IIIA	7 (16.7)	4 (17.4)	11 (16.9)
IIIB	6 (14.3)	1 (4.3)	7 (10.8)
IV	11 (26.2)	7 (30.4)	18 (27.7)
Histology			
adeno	19 (45.2)	14 (60.9)	33 (50.8)
signet-ring	12 (28.6)	6 (20.1)	18 (27.7)
mixed	11 (26.2)	3 (13)	14 (21.5)

[a]GC, gastric cancer; [b]GEJ, cancer of the gastroesophageal junction.

Evaluation of immunostaining. The analysis of the slides was carried out by two independent examiners who were blinded to the PCR results and clinical outcome of the patients. A subsequent review was performed by a pathologist. For evaluation of TKTL1 slides were examined for evidence of cytoplasmatic or nuclear staining. The cytoplasmatic

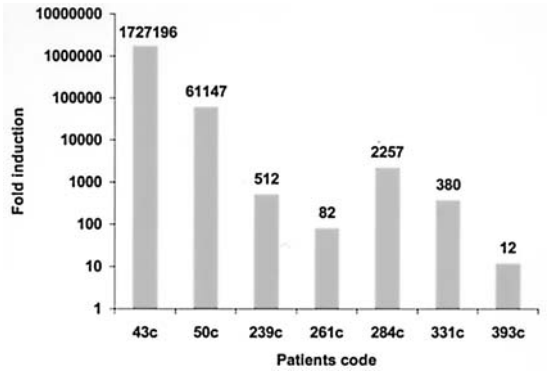

Figure 1. Expression levels of TKTL1 in real-time PCR. Data are presented as x-fold cycles compared to matched normal tissue with the x-axis in a logarithmic scale.

immunoreactivity was scored from 0 to 3. The scale was defined as follows: score 0 indicates 0 to 20%, score 1: 21 to 50%, score 2: 51 to 80%, and score 3: >80% of tumor cells that were stained for TKTL1. Strong positive staining (score 2 or 3) was considered as significant overexpression and noted as positive for statistical purposes. In addition, nuclear staining was noted as present or absent.

Statistical analysis. Data were collected with Microsoft Excel 2003®. For statistical analysis SAS-Software® (Version 8.2) was used. Fisher's exact test and χ^2 test were used to compare the prevalence of TKTL1 expression with clinico-pathological features. Statistical significance was defined as p-values <0.05. All data are presented as mean±standard deviation (SD) if not stated otherwise.

Results

Between December 1999 and June 2002 specimen were obtained from 65 consecutive patients with GC or cancer of the GEJ. Patients and tumor characteristics are shown in Table I.

Real-time PCR. A minimum of 3 cycles (8-fold) excessing the matched normal tissue was considered as significant overexpression of TKTL1 in cancerous tissue. Out of the 65 evaluated tumors, seven (10.8%) had a significant over-expression [GC: n=4 (6.2%), GEJ: n=3 (4.6%)]. One tumor from the GEJ had a very high overexpression with 172,7196 cycles compared to the corresponding normal tissue. The detailed description of the tumors with over-expression are shown in Fig. 1.

Immunohistochemistry of TKTL1. To determine the role of TKTL1 in tumor cell metabolism, immunohistochemical staining was performed to identify the presence of the enzyme. In 36.9% of the evaluated tumors, a predominantly cytoplasmatic staining with strong positive tumor cells for TKTL1 was detected. In the adjacent stromal tissue some lymphocytes showed immunoreactivity but not the stromal cells (Fig. 2A and B). In 20% of cases an additional strong nuclear expression was observed (Fig. 2B and C). Few normal gastric specimens showed only weak irregular cytoplasmatic staining. Specific results are shown in Table II.

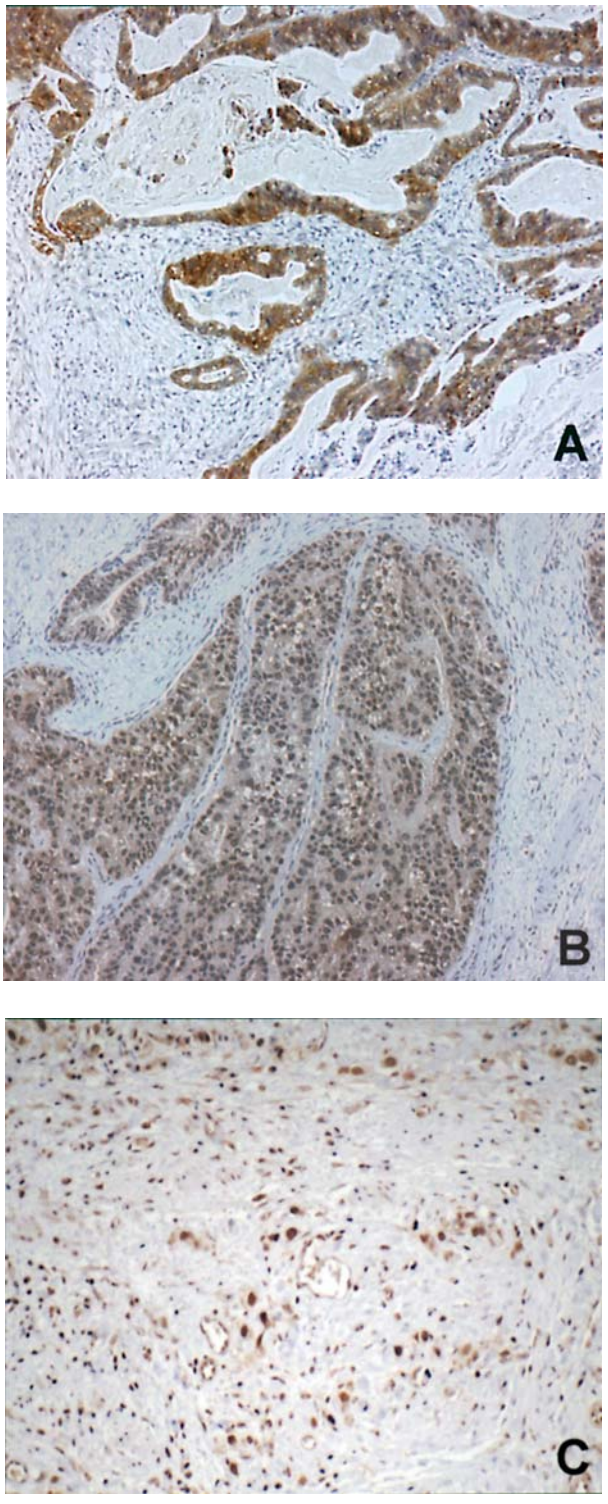

Figure 2. Immunohistochemical staining for TKTL1. Moderately differentiated adenocarcinoma from the GEJ. A strong cytoplasmatic staining is observed, while no staining exists in the nucleus or in the surrounding stromal cells (A). Moderately differentiated gastric adenocarcinoma. Cytoplasmatic and nuclear staining of carcinoma cells is observed (B). Original magnification: x50. Poorly differentiated adenocarcinoma from the GEJ. A strong nuclear staining of carcinoma cells is observed (C). Original magnification: x100.

Table II. Immunohistochemical expression of TKTL1.

	GC[a] n (%)	GEJ[b] n (%)	Both n (%)
Cytoplasmatic (expression score 2, 3)	15 (23.1)	9 (13.8)	24 (36.9)
Nuclear	6 (9.2)	7 (10.8)	13 (20.0)

[a]GC, gastric cancer; [b]GEJ, cancer of the gastroesophageal junction.

Correlation between different examination techniques. All cancer tissues with overexpression in real-time PCR also showed immunohistochemical staining for TKTL1.

Correlation with the clinicopathological features. A positive correlation of TKTL1 expression with poorly differentiated (G3) tumors (p=0.04) was demonstrated. Furthermore, a strong correlation between TKTL1 expression and the male gender (p=0.01) was observed. In the detailed analysis of the real-time PCR overexpression of TKTL1 we were unable to find any correlation to clinical or pathological features. The results are shown in Table III.

Table III. The relationship between TKTL1 expression in immunohistochemistry (IHC) and real-time PCR over-expression with clinicopathological features.

	IHC	PCR
Gender		
male	p=0.01	n.s.
female	n.s.	n.s.
Tumor site		
GC/GEJ	n.s.	n.s.
pT N M categories	n.s.	n.s.
Differentiation of tumor		
G3	p=0.04	n.s.
UICC-stage	n.s.	n.s.
Histology		
adeno	n.s.	n.s.
signet-ring	n.s.	n.s.
mixed	n.s.	n.s.

Discussion

TKTL1, a molecular marker in a subset of gastric cancer patients. In this study we demonstrate increased transketolase enzyme activity in a subset of patients with GC and cancer of the GEJ. The enzyme of relevance was TKTL1, which is over-expressed on mRNA and protein levels in a subset of the analyzed tumors. Excessively high values in some analysis suggest high enzyme activities in the respective tumors. The mRNA level were uniformly paralleled by immunohistochemical staining for the TKTL1. Using a novel anti-TKTL1 antibody, in 36.9% of the patients a strong immunohistochemical staining was detected. These findings demonstrate that TKTL1 upregulation on mRNA and protein levels is a relevant phenomenon in GC and cancer of the GEJ in a subset of tumors.

In gastric cancer patients, a correlation of TKTL1 to the male patients was shown. However, no evidence exists for a different distribution of human transketolases in respect of gender, neither are involved in the pentose phosphate pathway or the thiamine metabolism. Since the number of analyzed patients is limited, a gender specific TKTL1 expression should be evaluated in a larger series. The correlation of TKTL1 upregulation to poor differentiation of tumors suggests that the profound regulation disturbances in poorly differentiated tumors include alterations in the TKTL1 dependent pathways.

It is expected that molecular markers will have a major impact on the classification and therefore treatment and monitoring of cancer. In gastric cancer molecular markers might be helpful to identify subgroups of patients at high risk of tumor recurrence. Moreover, molecular markers could be useful for developing targeted therapy regimes. Especially for minimally invasive techniques such as endoscopic mucosal resection for early gastric cancer new clinical pathways have to be developed which consider extended tumor classification by specific variables describing the invasive potential of the respective tumor. In gastric cancer only few novel variables besides parameters like TNM-classification and resection margins are clearly established. Proposed relevant markers include several adhesion molecules (e.g. CD 44, E-cadherin) and other parameters (e.g. c-erbB-2, p53) (14-17). In glucose utilizing tumor metabolism the upregulation of TKTL1 plays a crucial role in different solid tumors as we have recently reported (13). The present evaluation suggests that the overexpression of TKTL1 is relevant in a subset of GC or GEJ tumors. It warrants further investigation in a larger group of patients and should be included in future molecular marker evaluations.

TKTL1 and tumor metabolism. With the PPP nucleic acids and nucleotides are synthesised through the degradation of glucose. The oxidative part of the PPP generates NADPH as an important reducing agent in cell metabolism. Both products are of the utmost importance in proliferating tissues and could be generated even under anaerobic conditions as frequently observed in fast growing cancer tissues. A crucial point in this metabolism is the thiamine (vitamin B1) dependence of the transketolases. Recently, it has been demonstrated *in vivo*, that the supplementation of thiamine significantly increased tumor growth (10). In patients with GC or cancer of the GEJ thiamine deficiency occurs frequently and therefore supplementation is routinely used pre-operatively or after gastrectomy as nutritional support. It is not determined yet, whether the benefits of this supplementation outweigh the risk of stimulation of tumor growth.

TKTL1 and the potential for therapy. The selective inhibition of transketolase might be a strategy for molecular targeted anticancer therapy. Pre-clinical trials achieved an inhibition of tumor proliferation by blockade of the transketolase enzyme reaction (7,8). Novel small-molecule inhibitors of transketolase have been identified which suppress tumor cell proliferation *in vitro* (18). The results of these studies have been attributed to an interference with the TKT enzyme. However, the data of this study offer another explanation. Tumors with upregulation of TKTL1 and absent TKT and TKTL2 expression are likely to be targeted through TKTL1 by these anti-TKT approaches. A detailed analysis of all TKT enzymes in intervention trials focusing on transketolase enzymes would be most interesting.

In this evaluation a considerable subgroup of patients with TKTL1 overexpression was identified. In future, these patients are of clinical interest for a novel molecular based inhibition of the transketolase enzyme reaction. Moreover, larger series of patients with gastric cancer have to be studied for a profound confirmation of our data. First however, the molecular basis of TKTL1 upregulation in different solid tumors has to be better understood.

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