Expression of Transketolase-like 1 Protein (TKTL1) in Human Endometrial Cancer

M. KROCKENBERGER, J.B. ENGEL, M. SCHMIDT, N. KOHRENHAGEN, S.F.M. HÄUSLER, Y. DOMBROWSKI, M. KAPP, J. DIETL and A. HONIG

Department of Obstetrics and Gynecology, University of Wuerzburg, Germany

Abstract. *Background: Malignant tumors metabolize glucose to lactate even in the presence of oxygen (aerobic glycolysis). The metabolic switch from oxidative glycolysis to non-oxidative fermentation of glucose and proteins performed by the tumor cells seems to be associated with TKTL1 and pAkt overexpression. Therefore the aim of the present study was to investigate the expression of TKTL1 and pAkt in human specimens of endometrial cancer as compared to benign endometrium. Additionally, expression of the glucose transporter GLUT1 was also investigated as aerobic glycolysis is associated with an increased need for glucose. Materials and Methods: Levels of TKTL1, pAkt, and GLUT1 expression were immunhistochemically evaluated on paraffin embedded biopsy material from 10 benign and 41 malignant endometrial tissue samples. TKTL1 mRNA levels in the endometrial cancer cell lines Ishikawa and HEC-1A were evaluated by RT-PCR. Results: Expression of TKTL1, GLUT1 and pAKT was significantly increased in endometrial carcinomas as compared to benign endometrial tissue. There was a significantly weaker TKTL1 expression in highly differentiated G1 tumors. In the human endometrial cancer cell lines Ishikawa and HEC-1A, TKTL1 mRNA was clearly detectable. Conclusion: The levels of TKTL1, GLUT1 and pAKT expression point to the glycolytic phenotype of malignant endometrial tissue. Given the pronounced TKTL1 expression across all different subtypes of endometrial cancer, this protein could serve as a target for future cancer treatments.*

Endometrial cancer is the most common type of uterine cancer worldwide (1). The incidence of endometrial cancer in women in the U.S. is 1-2%. The incidence peaks between the ages of 60 and 70 years old, but 2-5% of cases may occur before the age of 40 (1). Although the exact cause of endometrial cancer is unknown, increased levels of estrogen appear to promote tumorigenesis in endometrial cells (1). Aromatase enzymes, commonly expressed in fat tissue, convert androgen precursor molecules into estrogen, which explains the association of obesity with this malignancy. Other risk factors include diabetes and polycystic ovarian syndrome. All these conditions are frequently characterized by insulin resistance and high blood glucose levels. Malignant cells have an excessive need for glucose, which can be visualized by positron-emission tomography (2, 3). Therefore, the characteristic high blood glucose levels in patients with conditions such as diabetes and obesity may contribute to tumor progression and pathogenesis of endometrial cancer (4).

The relevance of glucose metabolism for cancer cell biology has been described and reviewed in previous articles (5-9). The anaerobic degradation of glucose in tumors despite the presence of oxygen is known as aerobic glycolysis or Warburg effect. Nobel laureate Otto Warburg detected this phenomenon some 80 years ago, due to the high levels of lactate tumor cells generated under aerobic conditions. Recently, the importance of this finding has been underlined by various studies, demonstrating that certain metabolic enzymes are up-regulated in malignant cells (10). In eukaryotic cells, glucose can be catabolized without oxygen *via* the Embden-Meyerhoff or the pentose-phosphate (PP) pathway. Enzymes that play a crucial role in the PP pathway for this kind of glucose metabolism are the transketolase enzymes. Preliminary data suggest that inhibition of transketolase enzyme reactions suppresses tumor growth and metastasis (11, 12).

Since energy production *via* oxygen-independent glucose catabolism is quite ineffective when compared to the oxidative phosphorylation pathway, cells utilizing glycolysis are dependent on elevated glucose uptake (13). Facilitated glucose uptake into cells is predominantly mediated by a family of glucose transport molecules known as GLUTs. These GLUTs are expressed in a tissue specific manner and

Correspondence to: Arnd Honig, MD, Department of Obstetrics and Gynecology, Josef-Schneider Str. 4, D-97080 Würzburg, Germany. Tel: +49 93120125253, Fax +49 93120125406, e-mail: arnd_hoenig@hotmail.com

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are overexpressed in many primary tumors (13-15). Goldman *et al*. demonstrated that GLUT1 and GLUT-8 are expressed in both human endometrium and endometrial cancer, with increased GLUT1 expression in dedifferentiated tumors (16). A step-wise progression in GLUT1 and GLUT-8 expression is observed as tumor histopathology deteriorates (16). Von Wolff *et al*. did not find GLUT-8 expression in benign endometrial tissue but GLUT1 and proposed glucose transporter expression as an essential part of endometrial differentiation and decidualization (17). We evaluated the extent of glucose transporter 1 (GLUT1) expression and its potential coexpression with TKTL1 and pAkt in benign and malignant endometrial tissue.

We were interested in pAkt because a loss of PTEN and subsequent activation of the PI3K/AKT pathway lead to a glycolytic phenotype in malignant glioma cells. These glycolytic glioma cells were able to migrate invasively and furthermore the inhibition of PI3K/pAkt in these cells substantially decreased their ability to migrate invasively (18).

In this study, we investigated three key proteins indicative of altered glucose metabolism in endometrial cancer cells: GLUT1, which enables cells to increase glucose uptake, TKTL1 and pAkt as molecules, whose expression has been associated with the glycolytic tumor phenotype.

Materials and Methods

Tissue. Investigations were approved by the Ethics Committee of the Medical Faculty of the University of Wuerzburg, Germany. A total of 41 endometrial cancer and 14 benign endometrial tissue samples from premenopausal women undergoing hysterectomy due to uterine fibroids were collected. Immediately after surgery, tumor specimens were formalin fixed and paraffin embedded.

Cell lines and culture conditions. The human endometrial cancer cell lines Ishikawa, HEC-1A and AN3-CA were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The cells were cultured in Dulbecco's modified Eagle's medium (PAA, Germany) supplemented with 10% fetal bovine serum (PAA) and gentamycin, at 37˚C in a humidified atmosphere of 5% CO_2.

RT-PCR. RNA was extracted from cells using the Qiagen RNeasy Mini Kit (Qiagen, Hamburg, Germany). Extracted nucleic acids were digested with DNaseI (DNaseI Qiagen Cat. No. 79254) for 15 min at room temperature. mRNA was transcribed with RevertAid H Minus First-Strand cDNA Synthesis Kit (Fermentas K1632). Set Go Taq (Promega, Mannheim, Germany) ready to use reagents were used for PCR experiments. Samples of 5 μg of cDNA were used for PCR. Two different sets of primers, designed specifically to amplify mRNA of human TKTL1, were used. Both gave identical results. The first set (TKTL1a) had the following sequences: upstream-primer 5'-ACAACGACCGATTTGTCCTC-3' (289-308), and downstream primer 5'-AGGTTGCCACAAGATTGTCC-3' (500-519). The second primer pair (TKTL1b) consisted of the upstream primer 5'-GATCCAGAGAATCCGGACAA-3' (273-292) and downstream primer 5'-CCAGACAGAGCCTTCTGAGG-3' (448-467).

Table I. *Semiquantitative immunohistochemical evaluation of TKTL1, pAKT and GLUT1 expression.*

	Malignant				Benign			
Grade	3	2	1	0	3	2	1	0
TKTL1	29	6	5	1	0	0	4	6
GLUT1	16	16	7	2	0	0	3	7
pAKT	18	10	5	8	0	0	3	7

Taq polymerase (Promega) was used at 1 U/μl with 5 mM dNTP and 25 mM $MgCl_2$. The PCR was run for 40 cycles and the temperatures were 96˚C, 60˚C (annealing), and 72˚C. The PCR products corresponding to the mRNA were 230 and 194 base pairs for the two different TKTL1 primer pairs respectively. PCR products were run on a 1% agarose gel and analyzed. RT-PCR was considered positive when a clear band of the expected size was visible on an ethidium bromide-stained gel.

Immunohistochemistry and antibodies.
Antibodies. Monoclonal antibody against TKTL1 was kindly provided by Johannes Coy, R-Biopharm, Darmstadt, Germany. The antibody (clone JFC12T10) was raised against a *C*-terminal fragment of the recombinant TKTL1 protein in mice (19). A rabbit polyclonal antibody detecting GLUT1 (ab53767) (Abcam, Cambridge, UK) was used. The antibody was diluted 1:100. A rabbit polyclonal antibody detecting pAkt 1 (ab61766) (Abcam) was used. The antibody was diluted 1:100 and to demask antigens samples were heated in citrate-buffer pH 6.0 in a microwave oven 2 times for 5 minutes at 750 W.

Immunohistochemistry. Serial sections were cut at 2 μm from the paraffin-embedded uterine tissue specimens and placed onto APES (3-amino-propyltriethoxy-silane; Roth, Karlsruhe, Germany) coated slides, dewaxed in xylene, rehydrated in graded ethanol and distilled water, and subjected to heat pretreatment for antigen unmasking by boiling in 0.2 M sodium citrate buffer (pH 6.0) 2 times (pAKT) and 3 times (GLUT1) for 5 min in a microwave oven (750 W/s). After irrigating in distilled H_2O, inhibition of endogenous peroxidases was performed by incubation of the sections for 10 min in H_2O_2 (3% in methanol). Slides were washed in PBS and incubated with 1% goat serum for 15 min. Subsequently, slides were incubated with the monoclonal mouse anti-TKTL1 antibody (clone JFC12T10), previously described by Coy *et al*. diluted in commercial antibody diluent (DAKO, Hamburg, Germany) at 1:200-1:400 depending on tissue conditions (19). After 45-60 min of incubation at room temperature slides were washed in PBS and incubated with biotinylated anti-mouse immunoglobulins (DAKO) and treated with streptavidin-peroxidase (DAKO) according to the manufacturer's protocol.

The sections were incubated with the primary antibody at the appropriate dilution, followed by the horseradish-peroxidase (HRP)-labeled secondary antibody (dilution 1:100, DAKO). 3,3'-Diaminobenzidine (Sigma, Deisenhofen, Germany) was used as the chromogen.

Statistical analysis. GraphPad Prism Version 4 was used for statistical analysis. The Mann-Whitney *U*-test for nonparametric Gaussian distributions as well the Spearman rank correlation were applied.

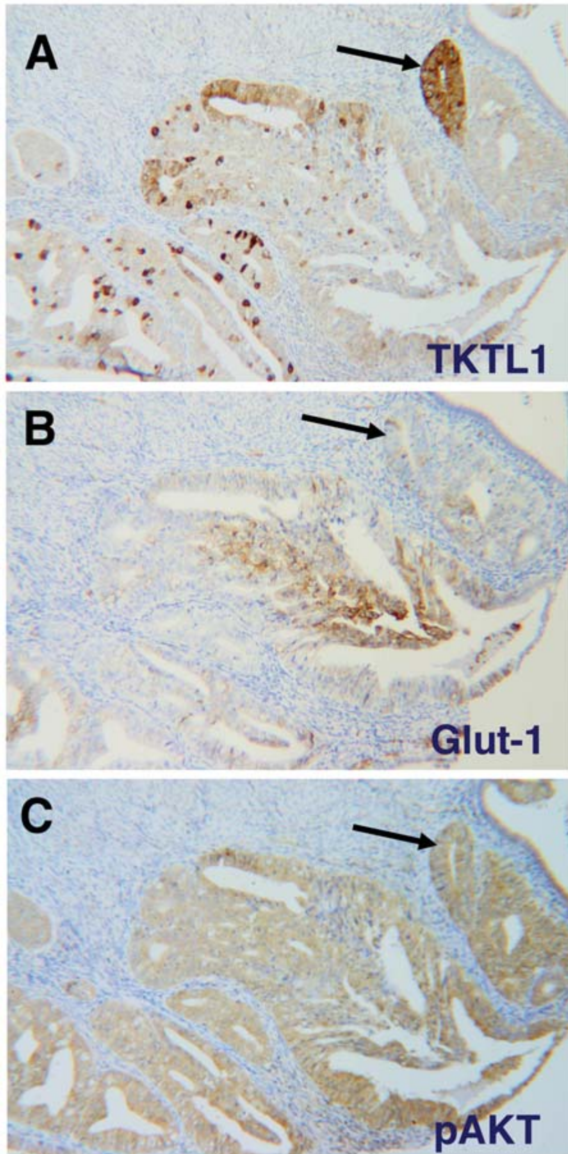

Figure 1. *A: TKTL1 expression was not homogenous in endometrial cancer. This sample exemplifies that within one tumor, areas with strong as well as weak TKTL1 staining could be detected. B: There was significant GLUT1 expression in endometrial cancer tissue. C: A relatively homogenous expression of pAkt was evident in the same endometrial cancer sample.*

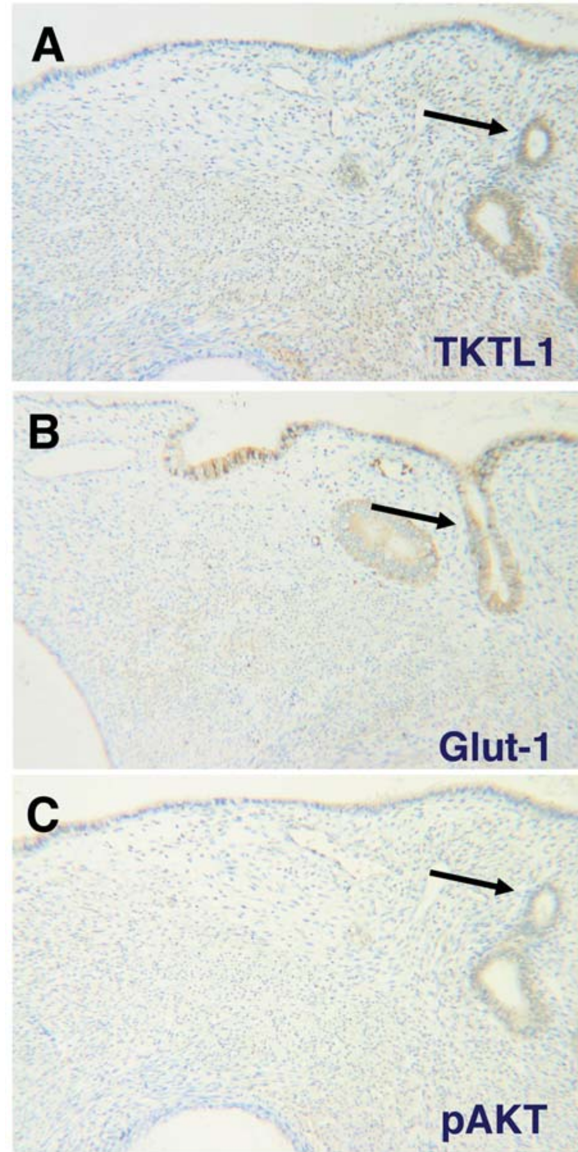

Figure 2. *A: In this benign endometrial sample of a postmenopausal woman hardly any TKTL1 expression was immunohistochemically detectable. B: In this benign endometrial sample of a postmenopausal woman only weak GLUT1 staining was detectable. C: Only weak expression of pAkt was detected in this sample of postmenopausal endometrial tissue.*

Results

Immunohistochemistry. The results of the semiquantitative evaluation of TKTL1 expression in benign and malignant endometrial tissue are summarized in Table I. Typical patterns of TKTL1 expression in normal endometrial tissue and carcinomas are shown in Figures 1 and 2. Remarkably some of the tumors showed a highly heterogenous expression pattern of TKTL1 (Figure 1A). Immunohistochemical staining revealed an elevated expression of TKTL 1 in 35 out of 41 carcinomas (semiquantitative immunohistochemical score 2-3) in comparison to 5 carcinomas and all benign control endometrial tissue samples with only moderate or none TKTL1 expression (semiquantitative immunohistochemical

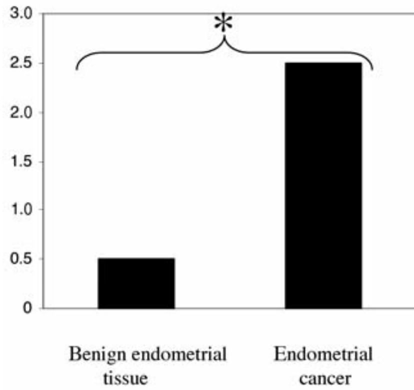

Figure 3. *TKTL1 expression was significantly up-regulated in endometrial cancer.*

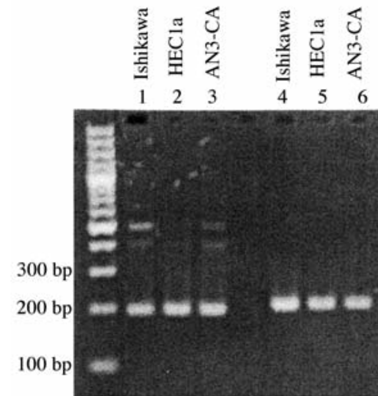

Figure 4. *TKTL1-specific mRNA expression. mRNA was clearly expressed in Ishikawa, HEC-1A and AN3-CA endometrial cancer cells. In lane 1-3, the smaller PCR products with 194 bp and in lane 4-6 the bigger PCR products with 230 bp are demonstrated.*

Table II. *Medians and means of TKTL1, pAkt, GLUT1 based on semiquantitative IHC scores.*

	Malignant	Standard deviation	Benign	Standard deviation
TKTL1 median	3		0	
TKTL1 mean	2.5	0.80	0.5	0.5
pAKT median	2		0	
pAKT mean	2.1	0.85	0.5	0.48
GLUT1 median	2		0	
GLUT1 mean	2.0	1.2	0.3	0.46

Table III. *Histological tumor subtype with regard to the level of TKTL1 expression expressed as semiquantitative immunohistochemical scores.*

	TKTL1 scores			
	0	1	2	3
Endometrioid adenocarcinoma	1	5	6	25
Clear cell carcinoma (2)	0	0	0	2
other (2)	0	0	0	2

score 0-1) (Table II, Figure 3). The difference in TKTL1 expression between benignant and cancerous tissue was highly statistically significant $p<0.01$.

TKTL1 expression in different histological subtypes of endometrial cancer. All carcinomas with less preferable prognosis like clear cell carcinoma and serous papillary endometrial cancer, although their number was small in our investigation, overexpressed TKTL1. The most prevalent histological subtype was the endometrioid endometrial adenocarcinoma. This subtype showed a slightly more heterogenous TKTL1 expression with 5 (22%) carcinomas with only moderate or none TKTL1 expession. Prognostic parameters of endometrial cancer patients were analyzed. Parameters of interest were initial tumor stage, histological grading, and age of patients at the time of diagnosis. In our study group a trend towards a correlation between TKTL1 expression and poor prognostic factors was detectable. Most undifferentiated grade 3 tumors showed a clear overexpression of TKTL1 with semiquantitative immunohistochemical TKTL1 scores of 2 or 3. Survival data were not available at the time of our investigation. Of the 41 carcinomas, 35 (85%) revealed a TKTL1 expression with scores of 2 or 3 and 6 (13%) showed a weaker TKTL1 expression with scores 1 or 0 (Table III). The mean age of the patients in the first group was 68 and 62 years in the second group. The data given in table IV indicate, that in general diagnosis of endometrial cancer is made at an early stage, and therefore due to the small number of advanced cases there was no use in correlating tumor stage with TKTL1 expression.

Correlation of TKTL1, pAkt and GLUT1. The semiquantitatively determined expression levels of TKTL1, GLUT1 and pAkt were correlated. The correlation between GLUT1 and pAkt did not reach statistical significance, although a trend towards a positive correlation was evident. The correlations between TKTL1 and GLUT1 as well as TKTL1 and pAkt did not reach statistical significance. All three antigens were significantly overexpressed in malignant tissue samples when compared to expression levels in benign tissue samples (Table III).

TKTL1-specific mRNA expression in endometrial cancer cell lines Ishikawa, HEC-1A and AN3-CA. RT-PCR was performed to confirm overexpression of the TKTL1 protein

Table IV. *Analysis of tumor stage and other prognostic markers in terms of TKTL1 expression level.*

TKTL1 score	Figo I %	Figo II %	Figo III %	Figo IV %	Benign histology	Mean histological grade	Mean age of patients (years)
2/3	25	6	3	0	0	1.6	68
0/1	4	1	0	0	14	1.4	62
Total	29	7	3	0	14		67

at the mRNA level. RT-PCR analysis in endometrial cancer cell lines Ishikawa, HEC-1A and AN3-CA showed a clear TKTL1 specific mRNA-expression. mRNA for TKTL1 was detected with two different oligo pairs (detecting different parts of the cDNA of TKTL1) depicted TKTL1a and TKTL1b resulting in two different PCR products of 230 bp and 194 bp respectively (Figure 4).

Discussion

To our knowledge, this is the first description of an overexpression of the TKTL1 protein in endometrial cancer suggesting that TKTL1 may play an important role in this cancer entity. Transketolase enzyme reactions of the non-oxidative part of the PP cycle enable oxygen-independent glucose degradation, and play a crucial role in nucleic acid ribose synthesis utilizing glucose carbons in tumor cells (20). Boros *et al*. showed that more than 85% of ribose recovered from nucleic acids of certain tumor cells derives directly or indirectly from the nonoxidative part of the pentose phosphate cycle (20).

Comin-Anduix *et al*. (21) demonstrated that transketolases determine cell proliferation in a tumor model. The authors demonstrated that the activation of transketolases by application of thiamine stimulates tumor growth. In general, the reactions of the pentose phosphate cycle lead to conversion of glucose to ribose for nucleic acid and also to NADPH synthesis. NADPH is an important reducing agent for nucleic acid synthesis. Proliferating tumor cells require both products and furthermore the pentose phosphate cycle allows anaerobic glucose degradation that has long been known as a characteristic feature of tumor cells (10). Due to rapid growth and a subesequently insufficient blood supply, tumors frequently show an anaerobic microenvironment. Interestingly, Gatenby and Gillies were able to demonstrate that anaerobic conditions are also present in premalignant lesions (22). Accordingly, it has been shown that tumor cells expressing TKTL1 can nonoxidatively degrade glucose to generate ATP (19). The importance of transketolases for tumor cell metabolism is underlined by the fact that the application of specific transketolase inhibitors induces a dramatic reduction in tumor cell proliferation (23).

As tumor tissue is characterized by repeated temporary decrease of oxygen pressure levels, the ability to utilize glucose in the absence of oxygen represents a selective growth advantage for tumor cells. Thus, Ramanathan *et al*. showed, that during malignant transformation tumor cells become more dependent on aerobic glycolysis and less dependent on mitochondrial aerobic generation of adenosine-triphosphate from glucose utilization (24). We demonstrated the expression of TKTL1 at the protein and mRNA levels in endometrial cancer. TKTL1 is also overexpressed in other malignant cells and is presumably involved in the metabolic switch leading to this glycolytic tumor phenotype (19).

Using an inhibitor of the mitochondrial respiratory chain it was demonstrated that a fully transformed tumor cell does not depend on this form of energy generation, because the inhibitor was not able to lower the ATP output of these cells. This insensitivity to a mitochondrial inhibitor is consistent with a predominant ATP generation through aerobic glycolysis. However, substances that impair aerobic glycolysis exhibited a profound decrease of ATP generation in tumor cells (5, 9, 25).

Another protein that seems to contribute to this metabolic phenotype by stimulating aerobic glycolysis in cancer cells is pAkt. pAkt regulates fundamental processes like metabolism and survival through phosphorylation-dependent inactivation of tumor suppressors and activation of trophic signaling. pAkt signaling regulates transport and metabolism of glucose and amino acids (26). Most importantly with respect to our investigation pAkt directs cells to undertake a metabolic conversion from oxidative phosphorylation to aerobic glycolysis (26). It is well known that pAkt is overexpressed in endometrial cancer, as the PTEN tumor supressor gene, which counteracts activation of Akt, is frequently deleted in this tumor entity (27). Accordingly, we found pAkt expression in malignant endometrial tissue to be increased when compared to the corresponding benign tissue. The metabolic conversion renders endometrial cell survival dependent on a continuous supply of extracellular nutrients like glucose. As a consequence up-regulation of GLUT1 in endometrial cancer might be a necessity for tumor cell survival. Aerobic glycolysis is less efficient than regular oxygen dependent glucose utilization but most probably faster, which might help rapidly proliferating tumor cells (28, 29). Furthermore GLUT1

expression correlates with prognosis in a range of tumors (30, 31). Regulation of glucose transport facilitator expression has been demonstrated in endometrial tissue and endometrial adenocarcinoma previously (32). The correlation of GLUT1 and pAkt expression in endometrial cancer did not reach statistical significance. Although a trend towards a coexpression of these antigens was evident. The majority of tumors were found to have up-regulated the TKTL1 protein and expressed the GLUT1 simultaneously, although this did not reach statistical significance as well. In both cases, this might be due to the rather small size of the study group.

Conclusion

TKTL1 is most probably contributing to the altered glucose metabolism of malignant tumors, which is conferring a selective growth advantage to tumor cells. A therapeutic intervention targeting the glycolytic tumor metabolism of a malignant endometrial cell has the potential to be effective without severely harming benign cells. Therefore, TKTL1 could serve as a target for future cancer treatments.

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