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Glucose Transporter-1 (GLUT-1) Immunoreactivity in Benign, Premalignant and Malignant Lesions of the Gallbladder

Mateja Legan · Špela Tevžič · Ana Tolar · Boštjan Luzar · Vera Ferlan Marolt

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Abstract GLUT-1 is a transmembrane glucose transport protein that allows the facilitated transport of glucose into cells, normally expressed in tissues which depend mainly on glucose metabolism. Enhanced expression of GLUT-1 can also be found in a large spectrum of carcinomas. This study aimed to investigate GLUT-1 expression in gallbladder tissue: from normal tissue samples, hyperplasias, low-grade and high-grade dysplasias to gallbladder carcinomas. In all, 115 archived samples of gallbladder tissue from 68 patients, presented after cholecystectomy, were immunohistochemically stained for GLUT-1. According to the intensity of GLUT-1 immunoreactivity, samples were divided into negative (stained 0–10% of cells stained), positive with weak to moderate (10–50%) and positive with strong (>50%) GLUT-1 expression. The GLUT-1 immunoreactivity of the samples showed a characteristic increase from premalignant lesions to carcinomas. Normal gallbladder tissue samples did not express GLUT-1 (100%). Weak expression was shown only focally in hyperplasias, but to a greater extent with low-grade dysplasias (20%), high-grade dysplasias (40%) and carcinomas (51.8%). Normal gallbladder tissue is GLUT-1 negative. GLUT-1 expression in carcinoma tissue is significantly higher than in dysplastic lesions. Strong GLUT-1 expression indicates 100% specificity for detecting gallbladder carcinomas. Therefore, GLUT-1 is a candidate as a diagnostic as well as a tissue prognostic marker in gallbladder carcinoma patients.

Keywords Diagnosis · Gallbladder adenocarcinoma · GLUT-1 · Immunohistochemistry · Prognosis

Abbrevations

GLUT-1	glucose transporter-1
No.	number
vs.	versus

Introduction

The growth of carcinoma cells is a time- and energy-consuming process. The faster and the more invasively the tumor grows, the more energy it needs, and this demand is fulfilled through an increased intake, utilization of glucose and its enhanced anaerobic metabolism [1]. It is a process that brings energy in an ATP form and is further processed in the growth and repair of tumor cells. It is a process mediated through glucose transporters, transmembrane proteins that under these special circumstances polymerase and assure facilitated glucose transport [2, 3].

GLUT-1 is a transmembrane transport protein that facilitates glucose transport into cells. It is normally expressed in the membranes of erythrocytes, endothelium of brain capillaries, perineurium, renal tubules and germinal centers of activated lymphatic tissues [4–6]. GLUT-1 has been shown to be occasionally present in benign changes of squamous epithelia and reactively changed epidermal cells: however, in most epithelial tissues GLUT-1 is absent or present in very small quantities, but its increased expression

M. Legan (✉) · Š. Tevžič · A. Tolar
Institute of Histology and Embryology, Faculty of Medicine,
University of Ljubljana,
Korytkova 2, SI-1000,
Ljubljana, Slovenia
e-mail: mateja.legan@mf.uni-lj.si

B. Luzar · V. F. Marolt
Institute of Pathology, Faculty of Medicine,
University of Ljubljana,
Ljubljana, Slovenia

occurs in a wide spectrum of malignomas [7, 8]. Reisser et al. [9] evaluated the expression of GLUT-1 in the development of squamous cell carcinomas of the head and neck. Noguchi et al. [8] searched for the presence of GLUT-1 in normal gastric mucosa and in gastric carcinomas. Haber [10] studied GLUT-1 expression in colorectal carcinomas, as well as in benign epithelia of the colon and adenomas. GLUT-1 expression is high in colorectal, head and neck, and gastric carcinomas, being absent or present in very small quantities in premalignant changes. Benign ovarian tumors and borderline non-invasive lesions in the study of Kalir et al. [11] did not show GLUT-1 staining, but borderline tumors were GLUT-1 positive in 80% and ovarian carcinomas in 96% of observed cases. GLUT-1 immunoreactivity has diagnostic value in benign, premalignant and malignant changes of the endometrium [12]: endometrial hyperplasias were always GLUT-1 negative, but atypical hyperplasias—connected with a high risk of development of endometrial carcinomas—stained positively in 71 %. Contrary to observations in squamous-cell carcinomas of the head and neck [13], colorectal carcinomas, carcinomas of the pancreas [14] and breast [15], in thyroid carcinomas a connection between enhanced GLUT-1 expression and malignant transformation was not found [16]. GLUT-1 was not a useful marker in distinguishing papillary thyroid carcinomas from benign lesions.

A gallbladder carcinoma is a malignancy with a short survival time, usually diagnosed late in the progress of the disease and many times incidentally, for example during a cholecystectomy because of gallstones. At times it is difficult to distinguish between high-grade dysplasia and early carcinomas, and moreover, apart from surgical treatment there is no successful adjuvant therapy for this type of malignancy. All these circumstances call for additional diagnostic (and prognostic) tools. Data on GLUT-1 expression in gallbladder carcinomas are scarce [17]. Diagnostic tools for distinguishing between high-grade dysplasias and already present carcinomas are rare, but the importance of such markers for an accurate and precise diagnosis, as well as the prognosis for the patient, is enormous. Moreover, in contemporary oncology, new therapeutic strategies are in progress, directed towards certain regulatory proteins of carcinogenesis and neoangiogenesis. In the case of a proven higher GLUT-1 expression and its role in the prognosis of gallbladder carcinomas, this transport protein could be a target for treatment with potential inhibitors of glucose transmembrane transport, which is in the experimental research phase [18].

The purpose of this study was to evaluate the expression of GLUT-1 in normal gallbladder tissue, hyperplastic, dysplastic and malignant lesions of the gallbladder. We expected the GLUT-1 expression in gallbladder carcinomas to be significantly stronger than in benign or premalignant lesions and that GLUT-1 could be a new diagnostic tool in distinguishing between morphologically dubious premalignant and malignant changes of the gallbladder.

Methodology

Patients and Tissue Samples

We performed a retrospective study of 115 gallbladder specimens from the archives of the Institute of Pathology of the Faculty of Medicine at the University of Ljubljana: 16 specimens of normal gallbladder tissue, 13 hyperplasias, 15 low-grade dysplasias, 15 high-grade dysplasias and 56 adenocarcinomas. The specimens were from 68 patients, operatively treated between 1998 and 2005. Cholecystectomy was performed, either because of suspected tumor lesions or because of gallstones. The post-operative histopathologic diagnoses of patients were in 56 cases gallbladder carcinoma and in 12 cases benign lesions. There were more tissue samples than patients because sometimes in a single gallbladder there were many clearly visible and detectably more pathohistological entities (e.g. area of hyperplasia and dysplasia in the same benign gallbladder; in 19 carcinoma cases, normal gallbladder mucosa tissue and/or well preserved dysplasia was obtained from the resection margin of the gallbladder carcinoma).

Immunohistochemistry

GLUT-1 expression was investigated using immunohistochemistry. Formalin-fixed, paraffin-embedded specimens were sectioned into 4 μm-thick sections. After deparaffinization, the slides were immersed in Dako REAL™ Peroxidase-Blocking Solution (EnVision Detection System, Dako, Denmark) to deplete the endogenous peroxidase. After treatment in the PBS-buffer (pH=7.2), the tissue was incubated with primary antibodies and immunostained with the polyclonal anti-GLUT-1 (Interchim Neomarkers, France) in a dilution of 1:300. Immunostaining was detected with the standard EnVision reagent—Dako REAL™ EnVision/HRP, rabbit, mouse (EnVision Detection System, Dako): a dextran polymer coupled with peroxidase molecules and secondary antibodies against rabbit and mouse immunoglobulins. Antigen visualization was achieved by applying a standard reagent, DAB+ Chromogen (EnVision Detection System, Dako, Denmark), consisting of 3.3′-diaminobenzidine tetrahydrochloride in an organic solvent. Then, the specimens were counterstained with hematoxylin. The specificity of the applied antibodies was checked with positive or negative controls. For the positive control we used a gastric adenocarcinoma with known strong GLUT-1 expression, while for negative the same tissue was treated without a primary antibody.

GLUT-1 Analysis

The analysis was performed by consensus of three observers (ML, ST and AT) without prior knowledge of the clinical follow-up data. Immunostaining of cell membranes of the tumor cells (or dysplastic, hyperplastic, or normal epithelia) for GLUT-1 was determined to be negative if less than 10% of cells were stained, weak to moderately positive for 10 to 50% staining, and strong GLUT-1 positive when more than 50% of cell membranes were stained. Sometimes we observed weak staining of the cytoplasm, but for GLUT-1 positive cells we counted only cells with concomitantly stained membranes. In staining evaluation we did not count those areas of the specimen where GLUT-1 is normally expressed (erythrocytes, perineurium).

Statistical Analysis

The SPSS 16.0 statistical package was used to determine descriptive statistics, mean values and standard deviations. For numeric variables we counted mean values with a t-test for independent samples; for attributive statistics we used a χ^2 test. The results were found significant at a p value$<$0.05.

Results

Of our 68 patients, 25 (36.8%) were males and 43 (63.2%) were females. The mean age of our patients was 65.7± 12.8 years (range 34 to 84 years) (Table 1). In the observed normal gallbladders, GLUT-1 immunoreactivity was not detected. Among hyperplastic gallbladder epithelia, the first GLUT-1 positive reaction appeared, increasing to positive expression in 20 % of low-grade dysplasia and 40% of high-grade dysplasia cases (Fig. 1a). However, in none of these cases GLUT-1 expression was strong. Strong GLUT-1 expression was detected in carcinoma cases (Fig. 1b): 33.9% of adenocarcinoma cases were strongly GLUT-1 positive next to 17.9% weak to moderately positive carcinomas; however, there were also GLUT-1 negative carcinomas—48.2% (Table 2). Particularly strong staining was seen in the perinecrotic areas of the neoplastic growths or in the depth of the tumor tissue.

Table 1 Gender and mean age of patients with gallbladder lesions

	Male (No.)	Female (No.)	Mean age (yrs)*
Normal gallbladder	7	9	63.1±12.4
Hyperplasia	6	7	65.3±12.0
LG dysplasia	4	11	61.7±12.2
HG dysplasia	3	12	66.7±11.2
Carcinoma	19	37	67.6±12.0

* mean value±SD

LG low-grade, *HG* high-grade, *No.* number, *yrs* years

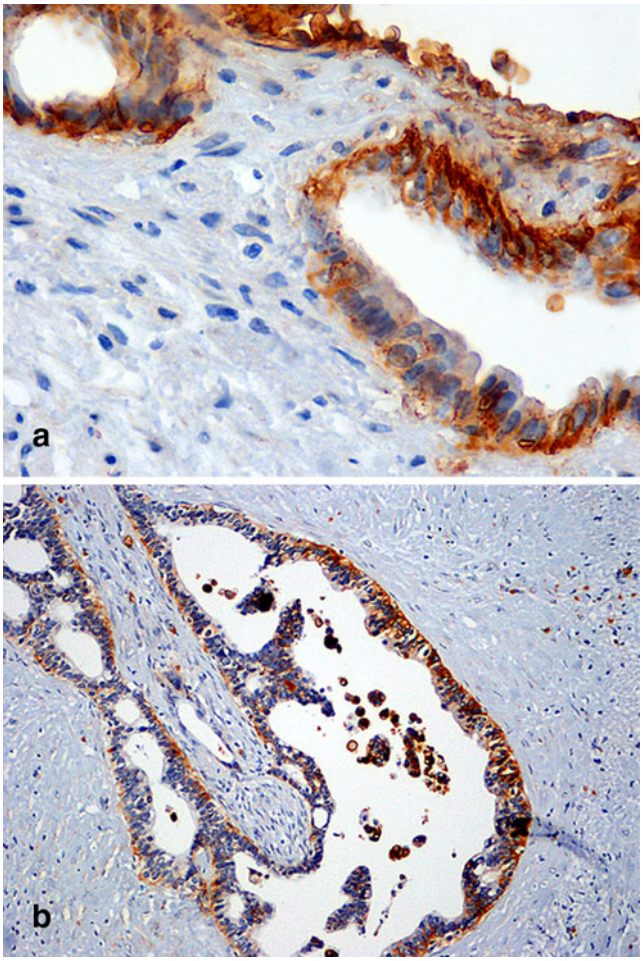

Fig. 1 GLUT-1 positive **a** high-grade dysplasia of gallbladder tissue (M×400), and **b** gallbladder carcinoma (M×200)

The differences in GLUT-1 expression (Table 2) between normal epithelium and hyperplastic epithelium, between hyperplastic epithelium and low-grade dysplasia, as well as between low- grade and high-grade dysplasia were not statistically significant (p=0.259 vs. 0.353 vs. 0.232). However, the difference in GLUT-1 expression between high-grade dysplasia and carcinoma was statistically significant (p=0.018).

We determined the grade of the tumors in all carcinoma samples. There were 10 adenocarcinomas of Grade I (well differentiated tumor), 17 Grade II (moderately differentiated) and 29 Grade III (poorly differentiated). Only 10% of the Grade I tumors were strongly GLUT-1 positive vs. 29.4% of Grade II, and 44.8% of the Grade III (Table 3), p=0.109.

Patients with tumors that showed strong GLUT-1 expression had a significantly shorter survival time than

Table 2 GLUT-1 expression in different gallbladder tissue forms

	GLUT-1 expression				No. of samples
	Negative		Positive		
	0%	< 10%	10–50%	> 50%	
Normal tissue	15 (93.8%)	1 (6.3%)	0 (0%)	0 (0%)	16
Hyperplasia	6 (46.2%)	6 (46.2%)	1 (7.7%)	0 (0%)	13
LG dysplasia	9 (60%)	3 (20%)	3 (20%)	0 (0%)	15
HG dysplasia	7 (46.7%)	2 (13.3%)	6 (40%)	0 (0%)	15
Carcinoma	3 (5.4%)	24 (42.8%)	10 (17.9%)	19 (33.9%)	56

LG low-grade, *HG* high-grade, *No.* number

patients whose tumors showed negative, weak or moderate GLUT-1 expression (6.3±7.0 months vs. 17.7±22.4 months, $p<0.001$) [19].

Discussion

Our study revealed that normal gallbladder tissue (epithelium and the underlying connective propria) does not express GLUT-1 immunoreactivity. With the progression of hyperplastic lesions to dysplasticity, GLUT-1 starts to be expressed from focal and weak reaction to gradually more pronounced. In high-grade dysplastic gallbadder tissue, more diffuse expression of GLUT-1 appeared, but only in gallbladder carcinomas strong GLUT-1 expression was observed. In all, 33.9% of carcinomas had strong GLUT-1 expression, and 17.9% moderate to weak; however, there was also a group of gallbladder carcinomas with negative GLUT-1 expression (48.2%).

Our research was retrospective in order to obtain a wide spectrum of tissue changes in the gallbladder along with information about survival (prognostic significance). Modern studies on GLUT membrane transporters are mostly based on immunohistochemical techniques [13, 20], which are superior to molecular PCR techniques, since GLUT is an ubiquitarian protein present merely in small quantities in all cellular membranes, but in significant quantities in cells and tissues dependant only on glucose metabolism (erythrocytes, perineurium). The aim of our study was to demonstrate a plausible higher expression of this protein in neoplastic and pre-neoplastic lesions of the gallbladder compared to normal tissue.

Our results have compelling diagnostic importance. Gallbladder carcinomas (all were adenocarcinomas) showed positive GLUT-1 immunoreactivity in 51.8%, with normal gallbladders being GLUT-1 negative. Immunoreactivity progresses through all stages—from hyperplasia, low-grade dysplasia end high-grade dysplasia to carcinomas.We revealed three diagnostic borders (limitations). Normal gallbladders are always GLUT-1 negative, taking into account slight GLUT-1 immunoreactivity in the hyperplastic epithelium (1 case). Gallbladder dysplasias have significantly higher GLUT-1 positivity (20–40%) than hyperplasias, whereas gallbladder carcinomas are the only lesions with strong GLUT-1 expression (33.9%). No benign gallbladder or its premalignant change showed strong GLUT-1 expression. This means that the specificity of strong GLUT-1 expression for detecting gallbladder carcinomas was 100%, but its sensitivity was 33.9%. In certain dubious cases where pathologists find it difficult to decide between high-grade dysplasias and carcinomas, strong GLUT-1 expression could facilitate the decision in the diagnosis of carcinoma; however, GLUT-1 negativity remains the diagnostic puzzle.

Our study deals with GLUT-1 expression in the gallbladder comprising a wide spectrum of normal tissue to carcinomatous lesions. It originates from the assumption that a carcinomatous process develops through several steps of progressive lesions [21–23] together with potentially early markers of premalignant and malignant changes. Several

Table 3 GLUT-1 expression with respect to carcinoma grade

	GLUT-1 expression				No. of cases
	Negative		Positive		
	0%	<10%	10–50%	>50%	
Grade I	0 (0%)	7 (70%)	2 (20%)	1 (10%)	10
Grade II	0 (0%)	9 (52.9%)	3 (17.6%)	5 (29.4%)	17
Grade III	3 (10.3%)	8 (27.6%)	5 (17.2%)	13 (44.8%)	29

No. number

studies on different neoplastic tissues have showed that GLUT-1 normally [8–15], but not always [16], accelerates expression in these tissues. GLUT-1 is the transmembrane transport protein for glucose, typical for hypoxic tumor tissues, and could be of extreme importance for their successful metabolism. GLUT-1 increases in quantity in the tumor cells of many tumor entities. It is foreseen that it is a marker of hypoxic tissue as well as of adaptation mechanism for tumor cells to be assured of glucose transport, which brings the necessary energy for growth and existence [3, 4]. For host tumor tissue which acts like a parasitic tissue, the successful model is a model that leads to invasion and metastasis. A number of studies [8, 11, 24] have showed a correlation between strong GLUT-1 expression and tumor invasion, leading to a shorter survival time of the patient.

Patients with strong GLUT-1 positive tumors had significantly shorter survival times than patients with negative or weak to moderate GLUT-1 positive tumors (6.3 vs. 17.7 months, $p<0.001$). Because strong GLUT-1 expression correlated with a poorer prognosis, we believed that GLUT-1 might also be a histological prognostic marker for patients with gallbladder carcinomas. Further research about gallbladder carcinomas would reveal important conclusions that should include prognostic factors (tumor invasion in local lymph nodes, surgical therapeutic margins, TNM classification) to determine whether multivariate analysis would show the importance of a singular prognostic factor for disease outcome. Our study including graduation of the analysed tumors determined the grade of the tumor as not significant in connection with survival ($p=0.461$, data not shown). We wondered if there was a correlation between GLUT-1 expression and tumor grade. Although we detected a trend of negative or weak GLUT-1 expression in carcinomas of the lower grade, the correlation was insignificant ($p=0.109$).

Our results are in accordance with Haber et al. [10], where strong GLUT-1 expression was detected in colorectal carcinomas; the patients with strong GLUT-1 expression had a worse prognosis than patients with weak GLUT-1 expression. As in our cases, there was no significant correlation between GLUT-1 and the degree of histological differentiation. A similar influence of GLUT-1 on the prognosis was demonstrated in ovarian carcinomas [11, 24], gastric cancer [8, 25], carcinomas of the cervix [26] and rectal carcinomas [20]. Strong GLUT-1 expression on the cell lines of pancreatic carcinoma [14] also had a positive connection with cellular invasion.

The discovery of enhanced GLUT-1 expression in premalignant and malignant gallbladder tissue opens up new therapeutic possibilities. Modern research on new anti-tumor agents already includes the first attempts to find GLUT-1 inhibitors that could block transmembrane glucose transport in neoplastic circumstances [18, 27]. Nevertheless, researchers are confronted with a difficult challenge since GLUT-1 has a crucial role in supplying the brain with glucose. Therefore it will be necessary to assure that any potential medicine will not be able to permeate the hemato-encephalic barrier to avoid a hypoglycemic effect on the central nervous system.

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