Pronounced Tumour Regression after Radiotherapy is Associated with Negative/Weak Glucose Transporter-1 Expression in Rectal Cancer

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Abstract. *The aim of this study was to assess glucose transporter-1 (GLUT-1) expression as a predictor of disease outcome in rectal cancer treated by preoperative radio- or chemoradiotherapy. Patients and Methods: Operative samples from 175 rectal cancer patients and 78 preoperative biopsies were analysed for GLUT-1 expression using immunohistochemistry. Forty-six patients received long-course radiotherapy, with/without chemotherapy and tumour regression grade was analysed in these specimens. Results: Negative GLUT-1 expression was seen in 25/78 (32%) of the preoperative biopsies and in 38/78 (49%) of the operative samples. There was no significant correlation of GLUT-1 with common clinicopathological factors. A trend towards longer disease-free survival (DFS) for the long-course radiotherapy group patients was seen with negative/weak GLUT-1 staining intensity (p=0.066) and excellent tumour regression grade (p=0.068) in operative samples. Disease-free survival (p=0.068) and disease-specific survival (p=0.024) of the patients with excellent tumour regression were longer than among the patients with moderate or less regression. Conclusion: A trend towards longer DFS among patients in favour of negative/weak GLUT-1 staining in the operative samples after long-course radiotherapy is demonstrated.*

Due to inadequate vasculature and rapid tumour growth, malignant tumours readily outgrow their blood supply, which leads to oxygen deficiency (1). Hypoxia starts a cascade of events including the stabilisation of hypoxia-inducible factor 1α (HIF-1α) (2). HIF-1α regulates several genes involved in *e.g.* angiogenesis, cell growth and glycolysis (2, 3). Hypoxia is associated with resistance to radiotherapy and chemotherapy (4, 5) and adverse disease outcome in malignant tumours (6).

In hypoxia, cells switch to using lactate production instead of oxidative phosphorylation from the TCA cycle for energy production, a phenomenon called anaerobic glycolysis or the Pasteur effect (7). Even in an environment with normal oxygen tension, cancer cells are inclined to use anaerobic glycolysis as a source of energy (Warburg effect) (8). In both cases, the uptake of glucose is strongly increased. Glucose is actively transported into the cell by transporter proteins (9). Glucose transporter-1 (GLUT-1) is the main carrier of glucose in malignant tumours (10). It is one of the downstream targets of HIF-1α (3). In addition to reduced oxygen concentration in hypoxia, GLUT-1 is also regulated by inhibitors of oxidative phosphorylation (11). GLUT-1 is widely expressed in tissue–blood barriers (10). It is overexpressed in several malignancies, *e.g.* carcinomas of the colon (12) and pancreas (13).

Radiotherapy can modify the tumour microenvironment (14). Both tumour and stromal cell sensitivity determine the extent of response to radiation (15), which can lead to tumour shrinkage, down-staging or even complete tumour elimination (16). Along with diminished tumour size, the need for oxygen and glucose would also be expected to decrease. Preoperative radiotherapy has been shown to down-regulate HIF-1α in rectal cancer (6). It can be hypothesised that preoperative radiotherapy also alters GLUT-1 expression in the tumour.

The current study was designed to evaluate the effect of preoperative radiotherapy or chemoradiotherapy on GLUT-1 expression in a series of 122 rectal carcinoma patients. A total of 78 pre-radiotherapy biopsies from the same patients,

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as well as 53 operative samples from non-irradiated rectal-cancer patients, were used as controls. GLUT-1 expression was analysed in relation to clinical data as well as the histopathological features of the tumours, including their regression grade after therapy and disease outcome.

Patients and Methods

Study population. This study was based on archival operative tumour specimens from 175 rectal cancer patients treated at Turku University Hospital according to the standard treatment protocols and 78 diagnostic biopsies from patients treated with preoperative radio- or chemoradiotherapy. The study population was described in detail in our previous reports testing other biomarkers in the same cohort (6, 17).

The patients were treated with short- (n=76) (5x5Gy) or long-course radiotherapy (50.40Gy) (n=46), determined by the stage of the tumour according to the standard treatment protocols and the judgement of the multidisciplinary team. Long-course radiotherapy was given with (n=37) or without (n=9) chemotherapy (fluorouracil or capecitabine). Postoperative adjuvant chemotherapy was given to patients with lymph node positive or high-risk lymph node-negative tumours, according to standard practice (18). As a control group (n=53), another series of rectal cancer patients who had not received any treatment prior to surgery was studied. Postoperative radiotherapy, chemoradiotherapy or adjuvant chemotherapy was given to eligible control group patients, when indicated. After completion of the treatment protocols, all patients were followed-up at the Department of Surgery. The mean follow-up time was 47 months (median 42 months; range 2-114 months).

Most of the tumours were typical adenocarcinomas (95%), a minor proportion being classified as mucinous carcinomas (5%). Out of the operations, 173/175 (99%) were macroscopically and 95/122 (78%) were microscopically radical. Anterior resection was performed in 94/175 (54%) of the operations. Vessel invasion was seen in 36/121 (30%) of the tumours. The number of examined lymph nodes was at least 12 in 87 (50%) of the operative specimens. There were 46/175 (26%) N1 and 25/175 (14%) N2 tumours. The most common sites of distant metastasis were liver, lung and suprarenal gland.

The study protocol was approved by the Ethics Committee of the Hospital District of Southwest Finland. The collection and use of archival tissue material was approved by the National Authority for Medico-Legal Affairs. The study was conducted in accordance with the Declaration of Helsinki.

Detection of GLUT-1 expression. GLUT-1 expression was analysed in all the preoperative biopsies (n=78) (from the preoperative treatment groups) and in all the tumour samples taken at surgery (n=175). The primary antibody used was antibody to human GLUT-1 rabbit anti-human GLUT-1 IgG, affinity pure; (Alpha Diagnostic International, San Antonio, TX, USA), diluted to 1:1000 concentration and counter-stained with Mayer's haematoxylin. To detect the immunoreaction, a Power Vision poly-horseradish peroxidase immunohistochemical detection system (Immunovision Technologies Co, Burlingame, CA, USA), was used.

Analysis of GLUT-1 expression. GLUT-1 staining was primarily membranous, but simultaneous focal cytoplasmic staining was seen along with strong membranous staining. The analysis was restricted to membranous staining only, by two approaches: the percentage of positive tumour cells and staining intensity. Staining intensity was graded as 0 if negative, 1 if weakly, 2 if moderately and 3 if strongly positive. The percentages were given scores as follows: 0 for 0%, 1 for 0-2%, 2 for 3-9%, 3 for 10-25%, 4 for 26-50% and 5 for >50%. The staining in the biopsies was assessed only as negative or positive, in addition to the intensity. For comparison of the biopsies with the operative samples, the scores of the operative samples 0-2 (corresponding to percentages<10%) were further combined into one group, negative, and scores 3-5 (corresponding to percentages >10%) into positive.

Analysis of the tumour regression grade. The tumour regression grade after long-course radiotherapy was classified in the HE-stained sections of 45 tumours into three categories (poor, moderate or excellent response), utilising the modified Rödel and Dworak scales (19, 20), as described in our previous report (17). To confirm the regression grade, a total of 2-8 separate histological slides were studied in the cases of moderate or excellent response.

Statistical analysis. All the statistical analyses were run using SPSS® (SPSS, Inc., Chicago, IL, USA) and STATA™ (Stata Corp., College Station, TX, USA) software packages (PASW for Statistics, version 17.0.2 and STATA/SE 11.0). Frequency tables were analysed using the chi-square-test, with the likelihood ratio or Fisher's exact test for the categorical variables. Differences in the means of the continuous variables were analysed using the Mann-Whitney test or Kruskal-Wallis test for two and multiple independent samples, respectively. Inter-observer reproducibility of the GLUT-1 assessments was tested using regular (Cohen's) kappa and weighted kappa. Concordance of GLUT-1 expression between preoperative biopsies and operative samples was analysed using non-parametric paired-samples test (Wilcoxon signed ranks test). The inter-observer reproducibility of all the GLUT-1 assessments was moderate.

Univariate survival analysis for disease-free survival (DFS) and disease-specific survival (DSS) was based on the Kaplan-Meier method. To adjust for covariates, Cox proportional hazards regression model was used. All the statistical tests were two-sided and declared significant at p-value <0.05.

Results

General aspects of GLUT-1 staining. GLUT-1 expression with increasing intensity is displayed in Figure 1. The staining pattern was primarily membranous. The results of the GLUT-1 staining analysis with regard to the clinical characteristics of the patients are shown in Table I. There was no significant correlation of GLUT-1 expression with patient age, gender and treatment group, preoperative extent of tumour invasion, the type of operation, postoperative T, N or stage, tumour differentiation, tumour necrosis or vessel invasion.

GLUT-1 expression in the biopsies and respective operative samples. GLUT-1 expression in the biopsies and operative samples is shown in Table II. The positive GLUT-1 expression of 33/53 (62%) preoperative biopsies remained positive and that of 20/53 (38%) biopsies changed to

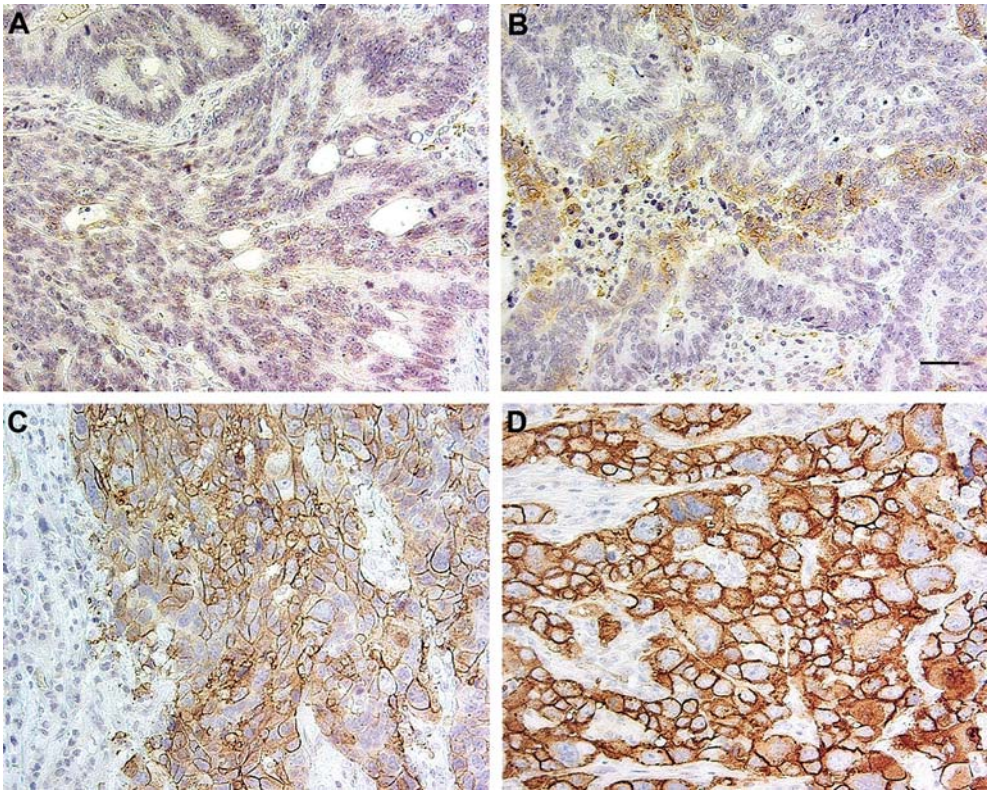

Figure 1. *Immunohistochemical staining of GLUT-1 in rectal cancer. (A) Negative, (B) weak, (C) moderate and (D) strong staining intensity. The subcellular localization of staining is predominantly membraneous.*

negative in the operative specimen. The negative GLUT-1 expression in 18/25 (72%) biopsies remained negative and 7/25 (28%) changed to positive in the operative sample (κ=0.301, CI (0.007–0.204), ICC 0.319).

DSS and DFS with regard to GLUT-1 expression. There were no significant differences in DFS or DSS in the short-course radiotherapy group in terms of GLUT-1 expression. In the long-course radiotherapy group, the patients who had negative/weak staining intensity in the operative specimen had a tendency towards better DFS (p=0.066) as compared to the patients with moderate/strong staining intensity. Figure 2 shows the DFS of the patients after long-course radiotherapy with regard to GLUT-1 staining intensity in the operative samples in univariate survival analysis.

Tumour regression after preoperative treatment. Tumour regression grade was analysed in 45 long-course radiotherapy group tumours. Two thirds (66%) of the tumours with an excellent response and half (50%) of the tumours with a poor/moderate response had negative/weak GLUT-1 expression (p=0.036). DFS (p=0.068) and DSS (p=0.024) of the patients that had excellent tumour regression were longer than those of the patients with moderate or poor response. There was one complete response (1/45, 2%) with no viable tumour cells left after preoperative treatment and no local relapses in the excellent response group.

Discussion

To the best of our knowledge, there are no prior reports comparing GLUT-1 expression in the diagnostic biopsies and respective operative samples after preoperative short-course and long-course radiotherapy. In the current study, GLUT-1 expression was positive in about half of the operative samples, well in line with previous studies in colorectal cancer (12) and other malignancies (13).

GLUT-1 staining in the preoperative biopsies and the corresponding operative samples was different in that two thirds of the biopsies (68%) but only half of the corresponding operative samples (51%) were GLUT-1-positive. Thus, there seemed to be a change in tumour GLUT-1 expression during the preoperative period. Interestingly, in an earlier study, the surgical procedure was shown to increase ischaemia and GLUT-1 expression levels significantly in rectal cancer (21). However, the biopsies

Table I. *GLUT-1 expression in the operative samples related to clinical characteristics.*

Variable	Positive n (%)	Significance
Treatment group		
Short-course RT	43/76 (57)	0.137
Long-course RT, no chemotherapy	6/9 (66)	
Long-course RT + chemotherapy	16/37 (43)	
Control group	30/53 (57)	
Preoperative T		
T1-T2	21/33 (64)	0.673
T3	29/54 (54)	
T4	24/46 (52)	
TX	21/42 (50)	
Postoperative stage		
I	22/43 (51)	0.813
II	31/56 (55)	
III	42/75 (56)	
No vital cancer	0/1 (0)	
Postoperative G		
G1	11/26 (42)	0.250
G2	68/114 (60)	
G3	13/29 (45)	
GX	3/6 (50)	

RT, Radiotherapy; T, tumour invasion; G, tumour differentiation.

Table II. *GLUT-1 expression in the biopsies and respective operative samples (n=78).*

Biopsy	Operative sample		κ [range]	ICC
	Positive n (%)	Negative n (%)		
Positive (n=53)	33 (62)	20 (38)	0.301	0.319
Negative (n=25)	7 (28)	18 (72)	[0.007; 0.505]	

κ, Kappa; ICC, intraclass correlation coefficient.

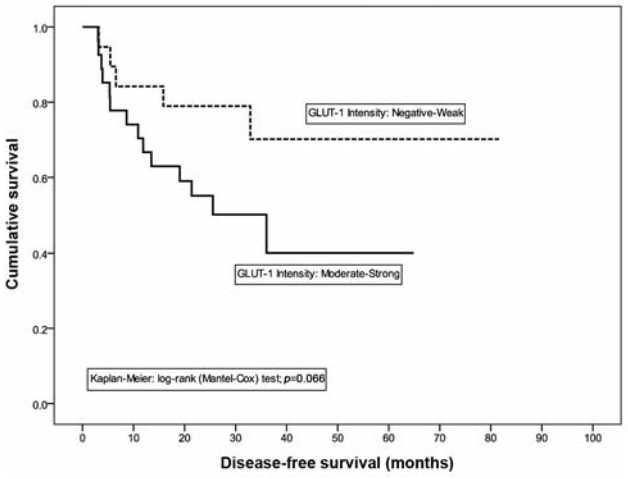

Figure 2. *GLUT-1 intensity related to DFS in univariate (Kaplan-Meier) survival analysis of the long-course radiotherapy group patients. (n=46).*

were taken immediately before the operation under anaesthesia, which may have had some effect on the results. In contrast, in the present series, GLUT-1 expression levels decreased when pre-radiotherapy biopsies were compared to the samples taken at operation. This implied that the preoperative treatment interfered with the tumour GLUT-1 expression. Since radiotherapy destroys tumour cells (16) and can affect the tumour microenvironment (14), it was not unexpected that preoperative treatment seemed to reduce hypoxia and hence, the GLUT-1 expression.

No statistically significant differences in either DSS or DFS with regard to GLUT-1 expression were found in the current study. However, excellent tumour regression was associated with negative/weak GLUT-1 expression and a tendency towards better disease outcome. Significantly, there were no local relapses after excellent tumour regression, which is of utmost importance considering the impact of local recurrences on the patient's quality of life and the risk of metastatic disease. After long-course radiotherapy, there was a trend towards better DFS among the patients, whose tumours had negative/weak GLUT-1 staining intensity compared to the patients, whose tumours had moderate/strong staining intensity. Probably due to the small number of samples, the difference in DFS did not reach statistical significance. However, Figure 2 shows a progressive difference in survival after the first months of follow-up. One possible explanation for the similarity of the survival curves at the start of follow-up could be that this patient population was at high risk of locally advanced disease. In some cases, overt metastatic disease was confirmed during the postoperative period and these patients did not recover postoperatively well enough to receive chemotherapy. Nevertheless, the tendency in the long-course radiotherapy group towards a favourable disease outcome of the patients with negative/weak GLUT-1 staining intensity is in parallel to the findings of a previous study showing an association of increased GLUT-1 expression in the pre-treatment biopsies with poorer response to chemoradiotherapy (22). Another study reported high expression of GLUT-1 to be an adverse prognostic factor in rectal cancer (23). However, it included only 6 patients who had received preoperative treatment. High GLUT-1 expression has been associated with poor prognosis in other malignancies as well (24).

Since malignant cells are more dependent on glycolysis as a source of energy than normal cells (8), and a link between GLUT-1 and chemoresistance has been shown (25), the inhibition of GLUT-1 could be an attractive target for therapeutic use in cancer. In fact, GLUT-1 gene silencing by

using siRNA has been demonstrated *in vitro* (26). In the future, incorporating new targeted therapies against glucose metabolism to preoperative chemoradiotherapy might enhance tumour shrinkage and thereby tissue oxygenation, which could possibly facilitate tumour operability and improve disease outcome.

In conclusion, preoperative treatment has an effect on the tumour's GLUT-1 expression and negative/weak GLUT-1 expression is linked to excellent tumour regression grade. There is a tendency towards better DFS after long-course radiotherapy, if GLUT-1 staining intensity in the operative sample is negative or weak.

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