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Expression of hexokinases and glucose transporters in treated and untreated oesophageal adenocarcinoma

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Summary. The aim of this study was to assess the expression pattern of the high glucose affinity glucose transporters GLUT 1, 2, 3, 4, 8 and 9 and of hexokinases I, II and III in newly diagnosed oesophageal adenocarcinoma by means of immunohistochemistry.

Twenty patients eligible to undergo primary surgery and 18 patients with incomplete pathological response following induction radio chemotherapy, all suffering from oesophageal adenocarcinoma, were included in the study. The intensity and amount of positive tumour cells in the immuno-reaction (histology score (Hscore)) for GLUT 1, 3, 4, 8 and 9 as well as for hexokinase I, II and III were assessed independently by two experienced observers, blinded to the clinical results.

In patients that underwent primary surgery, Hscores of GLUT8 (µ 6.7; sd3.3) and GLUT1 (µ 5.5; sd: 5.3) were significantly higher than Hscores of GLUT9 (µ 2.2; sd 1.5) and GLUT3 (µ 3.2, sd: 2.5). Hscores of hexokinase I (µ : 8.3; sd: 4.3), II (µ 5.5, sd: 4.0) and III (Ì 1.5, sd: 0.7) were all significantly different from each other ($p<0.04$). In patients that underwent radio-chemotherapy prior to surgical tumour resection, µ Hscores were 6.9 (sd: 4.4) for GLUT1, 6.8 (sd: 5.3) for GLUT3, 5.9 (sd: 4.2) for GLUT8, 3.4 for GLUT9 (sd: 2.7) and 2.3 (sd: 3.6) for GLUT 4. Hscores of GLUT1 and GLUT3 were significantly higher than Hscores of GLUT4. Finally, Hscores of patients with radio-chemotherapy for GLUT3, hexokinase II and III were significantly higher when compared to patients that underwent primary surgery.

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Introduction

Oesophageal adenocarcinoma is an increasingly common cancer with a poor prognosis. In the last 30 years, the rates of oesophageal adenocarcinoma have quadrupled, with a greater increase in men than women, and the 5 year survival remains less than 14% (Enzinger and Mayer, 2003; Jemal et al., 2004). Accurate staging of adenocarcinoma is important since survival, optimal management and degree of responsiveness to chemoradiation closely correlates with tumour, nodal and metastasis stage. Fluoro-deoxyglucose positron emission tomography (FDG-PET) is routinely performed for staging purposes in patients suffering from oesophageal adenocarcinoma since this imaging technique more accurately identifies non-nodal distant disease when compared to morphological imaging, e.g. CT (Annovazzi et al., 2003).

FDG PET imaging is based on the concept that malignant cells show increased glucose uptake and glycolysis (Warburg, 1956). Increased glucose uptake and glycolysis by tumour cells are due to increased numbers of membrane-bound glucose transporters, respectively, the GLUT/SLG2A family of glucose/polyol transport facilitators that are subdivided into three classes, and of glycolysis rate-limiting hexokinase enzymes (Joost and Thorens, 2001; Wood and Trayburn, 2003; Maria et al., 2005).

The aim of this explorative study is to assess the expression pattern of the high glucose affinity GLUTs, GLUT 1, 2, 3, 4, 8 and 9 and of hexokinases I, II and III

in adenocarcinoma of the oesophagus by means of immunohistochemistry. In addition, we also wanted to assess the influence of previous radio chemotherapy on the expression pattern of GLUTs and hexokinases in oesophageal adenocarcinoma.

Materials and methods

Patients

Thirty-eight consecutive patients suffering from oesophageal adenocarcinoma that either were eligible to undergo primary surgery (n, number of patients = 20) or induction radio-chemotherapy followed by surgery with curative intent but presented with an incomplete pathological response (n=18) were included in the study. For staging purposes, all patients underwent a barium contrast study, endoscopy, bronchoscopy, an endoscopic ultrasound examination, CT-scan and in a limited number of cases also an FDG-PET examination. The American joint Committee on Cancer version 6 TNM criteria were used for stage delineation. Patients presenting with cTis, $cT_{1-3}N_0M_0$ or $cT_{1-2}N_1M_0$ disease as defined by routine staging underwent surgery alone. Patients presenting with $cT_4N_0M_0$ or $cT_{3-4}N_1M_0$ disease as defined by routine staging underwent neo-adjuvant radio-chemotherapy followed by surgery. Radiotherapy included 20 fractions of 1.8 Gy/fr adding up to a cumulative dose of 36 Gy administered over a period of 4 weeks. Chemotherapy consisted of 800 mg/m^2 of 5-Fluouracil administered on day 1-4 and day 22-25 and of 80 mg/m^2 of cisplatinum administered on day 1 and day 22.

In patients that underwent primary surgery, additional information was obtained on alcohol and cigarette abuse and the distance of the primary tumour from the teeth row defined. The distance from the row of teeth was subsequently categorized into either upper- (from 0-25 cm) or mid-distal (25-40 cm).

Immunohistochemistry

Routinely processed, formalin fixed, paraffin embedded surgical pathology specimens from adenocarcinoma of the oesophagus were examined. Sections of 4 µm thick were mounted on SuperFrost® microscope slides (Menzel-Glaser, Braunschweig, Germany), which were deparaffinized in xylene and rehydrated in a downgraded series of ethanol. After flushing in water, heat induced antigen retrieval was performed for 20 minutes with the following buffer (EDTA pH = 8.0 or CIT pH = 6.0), cooled down for 20 minutes and then flushed in water for 10 minutes. The endogenous peroxidase present in tissue was blocked for 5 minutes with H_2O_2 (DAKO , Glostrup, Denmark) on each tissue slide. GLUT and hexokinase targeting antibodies were than incubated for 1 hour; the corresponding dilution factors (primary antibody diluted in 1%BSA/TBS) are indicated in table 1. After washing, the sections were then incubated for 30 minutes with a labelled polymer-HRP anti-rabbit secondary antibody (DAKO, Glostrup, Denmark). We used the chromogen 3,3-diaminobenzidine+ (DAKO, Glostrup, Denmark) for 10 minutes to visualize the signal into brown. After washing, the sections were counterstained with hematoxylin.

TRIS-buffered saline instead of the primary antibody was used as negative control on each slide in order to exclude false positive responses from non-specific binding of the secondary antibody. Prior to staining the surgical resected specimens, an isotype control was performed to estimate the non-specific binding of target primary antibodies to cell surface antigens. Non-specific binding is due to Fc receptor binding or other protein-protein interactions.

Immunohistochemical analysis

The intensity and amount of positive tumour cells in

Table 1. The different antibody dilutions and heat induced pre-treatment methods needed for the immunostainings.

Antibody	Company	Pretreatment	Dilution	+ control tissue
GLUT 1	DAKO	EDTA	1/100	internal RBC
GLUT 2	a-diagnostics international	CIT	1/50	LIVER
GLUT 3	santa cruz biotechnology	CIT	1/50	TESTIS
GLUT 4	santa cruz biotechnology	EDTA	1/100	PLACENTA
GLUT 8	a-diagnostics international	CIT	1/10	TESTIS
GLUT 9	a-diagnostics international	EDTA	1/25	PANCREAS CA
HK I	santa cruz biotechnology	EDTA	1/500	LIVER
HK II	santa cruz biotechnology	EDTA	1/100	LIVER
HK III	santa cruz biotechnology	EDTA	1/400	LIVER
Ki-67	neomarkers	CIT	RTU	

For each antibody the correct positive control tissues were used to optimize the immunostainings. These tissues were stained together with the adenocarcinomas of the oesophagus to verify the staining procedure. EDTA: ethylene diamine tetra acetaat, CIT: citrate, RTU: ready to use, RBC: Red Blood Cells.

the immuno-reaction were scored independently by two experienced observers, blinded to the clinical results. The percentage of tumour cells that were positive on the immuno-reaction were scored as follows: 0% (score 0), 0-20% (score 1), 20-40% (score 2), 40-60% (score 3), 60-80% (score 4) and 80-100% (score 5). Intensities of staining were categorized as absent (score 0), faint (score 1), average (score 2) or strong (score 3). Positive tumour cells were counted per high-power field (final magnification, 400x). An estimation of intensity and % positive tumour cells was made after counting ten high-powerfields. A final score was calculated as following: Hscore=[(a1xi1)+(a2xi2)]/2, where i=the score of intensity, a=the score of amount tumour cells that stained positive and 1 and 2 refer to the scores of the two observers.

Statistical analysis

SPSS version 12.0 was used for statistical analysis. Normalcy of Hscores distribution was assessed using the Kolmogorov-Smirnov test. Differences in hexokinase or GLUT Hscores were assessed using the Friedemann test or ANOVA with posthoc Bonferroni correction. Differences in Hscores between treated and untreated tumours were assessed using an unpaired two-tailed Wilcoxon test. A possible correlation between Hscores of various assessed GLUTs and hexokinases was assessed using the Pearson-correlation test or Spearman-rank correlation test and scatter plot analysis. Reproducibility of histological scoring was assessed by means of intra-class correlation analysis. The significance level used was <0.05.

Results

Clinical findings

Patient characteristics of patients that underwent primary surgery are presented in table 2 and those with neo-adjuvant radio-chemotherapy in table 3. Median age was 62.0 years (range: 38.0–84.0 years). Four patients suffered from stage I disease, 16 patients from stage II disease, 15 patients from stage III disease and 3 patients from stage IV disease.

Reproducibility assessment of the scoring technique

The scoring methodology used proved highly

Table 2. Description of the clinicopathological findings for the population with surgery alone.

Clinicopathological findings (PRIMARY SURGERY)	Case
Gender	
men	18
women	2
Age	
Mean (years)	63.1
Range (years)	38.0-84.0
Alcohol	
No alcohol	11
Alcohol use	9
Nicotine	
No smoking	11
Smoking	9
Distance from the row of teeth	
Upper+middle	7
Distal	13
Depth of invasion primary tumour	
PT1	3
PT2	9
PT3	8
Regional lymph Nodes	
PN0	10
PN1	10
Distant Metastasis	
PM0	19
PM1	0
PM1a	1
PM1b	0
TNM staging	
Stage I	3
Stage IIA	7
Stage IIB	3
Stage III	6
Stage IVA	1

Table 3. Description of the clinicopathological findings for the population with neo-adjuvant radiochemotherapy before surgery.

Clinicopathological findings (neo-adjuvant RT/CT)	Case
Gender	
men	17
women	1
Age	
Mean (years)	62.9
Range (years)	48.0-77.0
Distance from the row of teeth	
Upper+middle	4
Distal	14
Depth of invasion primary tumour	
PT1	1
PT2	3
PT3	14
Regional lymph Nodes	
PN0	5
PN1	13
Distant Metastasis	
PM0	16
PM1a	1
TNM staging	
Stage I	1
Stage IIA	4
Stage IIB	2
Stage III	9
Stage IVA	2

reproducible (intra-class correlation analysis for intra- and inter-observer variability, respectively r=0.94 (p=0.001) and r=0.90 (p=0.001)).

Results obtained in group 1 (20 patients that underwent primary surgery)

Representative staining results are shown in Figure 1.

Mean Hscores for GLUT expression in decreasing order of magnitude were respectively 6.7 for GLUT8 (range 0-12.5; sd: 3.3), 5.5 for GLUT1 (range 0.5 - 15; sd: 5.3), 4.3 for GLUT4, (range 0-13.5, sd: 4.2), 3.2 for GLUT3 (range 0.5-9, sd: 2.5) and 2.2 for GLUT9 (range 0-7; sd: 1.5). None of the tumours stained positive for GLUT2. Hscores of GLUT8 and GLUT1 were significantly higher than Hscores of GLUT9 and GLUT3.

Mean Hscores for Hexokinase expression were respectively 8.3 for hexokinase I (range 1.5-15; sd: 4.3), 5.5 for hexokinase II (range 0.5-15, sd: 4.0) and 1.5 for hexokinase III (range 0.5-3.5, sd: 0.7). Hscores of

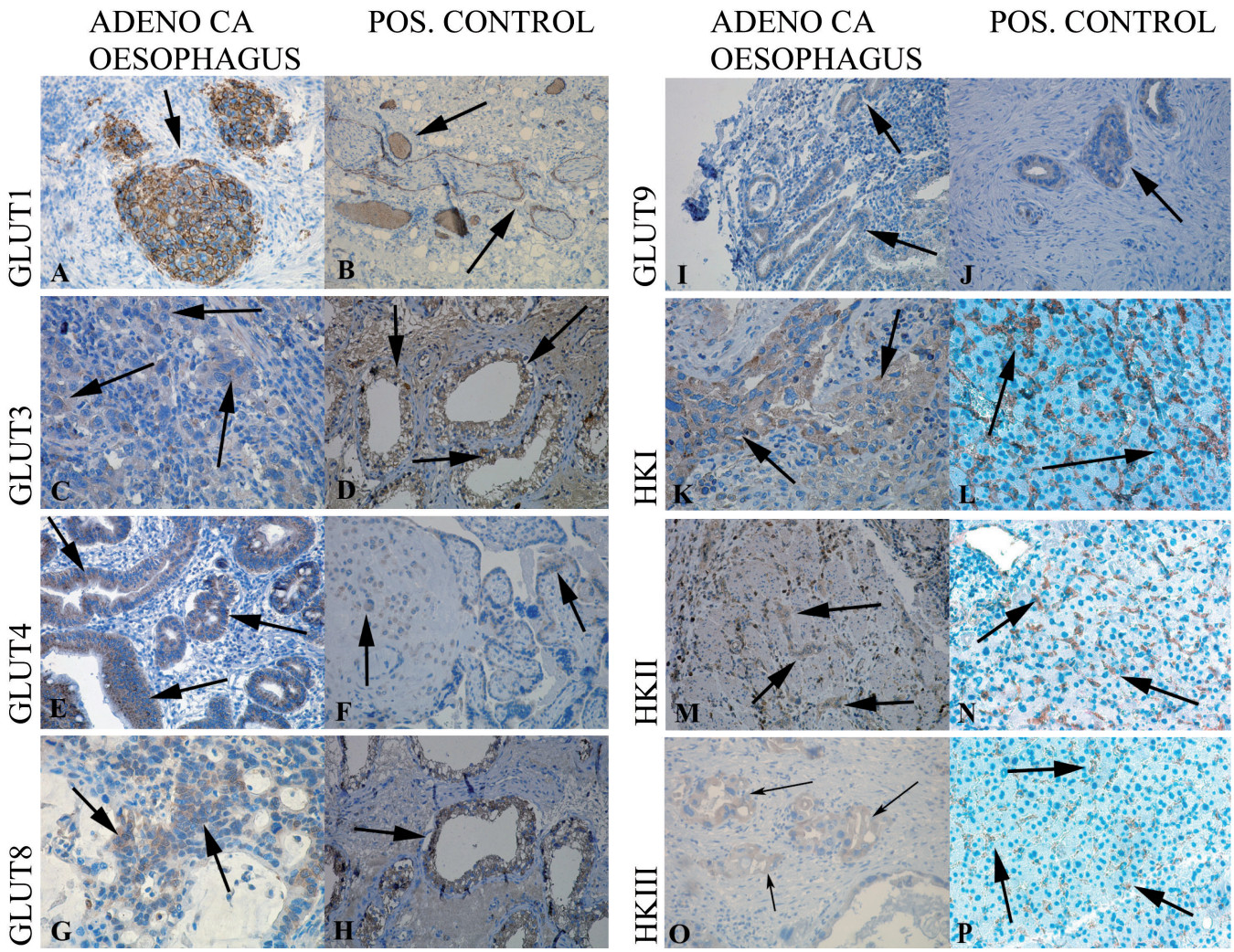

Fig. 1. A: arrows indicating GLUT1 positive tumour cells. **B:** arrows indicating positive internal erythrocytes and perineurium of the nerves. **C:** arrows indicating GLUT3 positive tumour cells. **D:** arrows indicating positive epithelial cells lining the ducti in testis tissue. **E:** arrows indicating GLUT4 positive tumour cells. **F:** arrows indicating positive syncythiotrophoblastic, cytotrophoblasts and dicidual cells present in normal placenta tissue. **G:** arrows indicating GLUT8 positive tumour cells. **H:** arrows indicating epithelial cells lining the ducti in testis tissue. **I:** arrows indicating GLUT9 positive tumour cells. **J:** arrows indicating positive cells in islets of Langherhans in pancreatic tissue. **K:** arrows indicating HKI positive tumour cells. **L:** arrows indicating positive sinusoids in normal liver tissue. **M:** arrows indicating HKII positive tumour cells. **N:** arrows indicating positive sinusoids in normal liver tissue. **O:** arrows indicating HKIII positive tumour cells. **P:** arrows indicating positive sinusoids in normal liver tissue. A, D-F, H-J, L-P, x 20: B, x 10; C, G, K, x 40.

hexokinase I, II and III were all significantly different from each other($p<0.04$).

GLUT3, and hexokinase III Hscores were significantly higher in those patients that had a history of cigarette abuse (n, number of patients = 9) versus those patients that had not (n=11), respective mean Hscores 4.4 (sd: 2.9) versus 2.2 (sd: 2.2) for GLUT3 ($p=0.04$) and 1.9 (sd: 0.9) versus 1.1 (sd: 0.4) for hexokinase III ($p=0.02$).

In patients that had a previous history of alcohol abuse (n=9), GLUT3 Hscores proved significantly higher when compared to those that had not (n=11), respective means Hscore of 4.3 (sd: 2.9) versus 2.1 (sd: 1.3) ($p=0.009$).

Hexokinase I and II Hscores proved significantly higher in tumours located in the upper and middle part of the oesophagus (n=7) when compared to those located distally (n=13), respective mean Hscores of 11.0 (sd: 3.2) versus 6.7 (sd: 4.2) for hexokinase I ($p=0.02$) and 8.6 (sd: 4.4) versus 3.8 (sd: 2.7) for hexokinase II ($p=0.008$).

Finally, GLUT and hexokinase I, II and III Hscores were not significantly different in those patients that presented with lymph-node involvement (n=10) versus those that did not (n=10), nor could a relationship with the degree of differentiation be documented.

Of interest, GLUT 8 Hscores were significantly correlated with GLUT3 ($r=0.662$, $p=0.01$) and GLUT 4 Hscores ($r=0.476$, $p=0.03$). There was no relationship between tumor stage and GLUT or hexokinase Hscores.

Of interest, pTNM data showed in half of the patients with T3/T4 tumors lymph node metastases. If these had been detected preoperatively, patients would have received neoadjuvant therapy. Comparisons of Hscores in this subgroup of patients to the subgroup of remaining patients that underwent primary surgery proved not significantly different ($p> 0.4$).

Results obtained in group 2 (18 patients that underwent radio-chemotherapy prior to surgical removal of the tumour)

Tumor regression grades (TRG) according to the scoring system developed by Mandard et al. were TRG 2 (presence of rare residual cancer cells scattered through the fibrosis) in 5 patients, TRG 3 (an increase in the number of residual cancer cells, but fibrosis still predominates) in 4 patients, TRG 4 (residual cancer outgrowing fibrosis) in 7 patients and TRG 5 (absence of regressive changes) in 2 patients (Mandard et al., 1994).

Mean Hscores for GLUT expression in decreasing order of magnitude were respectively 6.9 for GLUT1 (range 1.5-15; sd: 4.4), 6.8 for GLUT3 (range 0.5 to 15; sd: 5.3), 5.9 for GLUT8, (range 0 to 13.5, sd: 4.2), 3.4 for GLUT9 (range 0 to 9; sd: 2.7) and 2.3 for GLUT4 (range 0 to 15; sd 3.6). None of the tumours stained positive for GLUT2. Hscores of GLUT1 and GLUT3 were significantly higher than Hscores of GLUT4.

Mean Hscores for hexokinase expression were respectively 9.4 for hexokinase I (range 3 to 15; sd: 4.3), 8.6 for hexokinase II (range 1 to 15, sd: 4.4) and 3.8 for hexokinase III (range 0.5 to 15, sd: 3.3). Hscores of hexokinase I and II were not significantly different. However, Hscores of both hexokinase I and II were significantly higher than those obtained for hexokinase III.

Finally, GLUT9 Hscores proved significantly correlated with GLUT8 and GLUT1 Hscores, respective r-values = 0.63 ($p=0.05$) and $r=0.7$ ($p=0.01$).

Differences between group 1 and group 2

Hscores of patients treated with radio-chemotherapy (group 2) for GLUT3, hexokinase II and hexokinase III were significantly higher when compared to Hscores obtained in group 1 ; respectively 6.8 (sd: 5.3) versus 3.2 (sd: 2.5) for GLUT3 ($p=0.008$), 8.6 (sd: 4.4) versus 5.5 (sd: 4.0) for hexokinase II ($p=0,0310$) and 3.8 (sd: 3.3) versus 1.5 (sd: 0.7) for hexokinase III ($p=0.005$).

Discussion

GLUT1 is the human erythrocyte glucose transporter which is also localized to the perineurium, micro-vessels of the brain, placental trophoblasts, renal tubules and germinal centres in reactive lymph nodes. GLUT1 expression has been previously demonstrated in a wide variety of human tumours (Cantuaria et al., 2001; Furudoi et al., 2001; Sakashita et al., 2001; Tohma et al., 2005), including oesophageal carcinoma. In a series of 44 squamous oesophageal carcinoma, Kato et al. (2002) found that most of the tumours studied had some GLUT1 immuno-reactivity and that the percentage of positive cells was associated with tumour aggressiveness. In a series of 63 patients suffering from squamous oesophageal carcinoma, Tohma et al. (2005) found that there were two different patterns in positive staining, that is, weakly positive and strongly positive. The authors used the percentage of strongly positive tumour cells as an index for the evaluation of GLUT1 immuno-histochemical staining, as in their series the total percentage of positive cells was high in most cases. To date, only one study has addressed the presence of GLUT1 in adenocarcinoma of the oesophagus, respectively the study by Westerterp et al. (2007). In this series of 26 patients, GLUT1 expression proved negative to weak in 16 out of 26 patients studied and strong in the remaining 10 patients. Similar to their results, in our study the expression pattern of GLUT1 proved widely variable both in terms of the intensity of staining and in terms of the percentage of positive cells as evidenced by the wide range of Hscores found. In addition to GLUT1 expression, our series also documents for the first time the expression of various other high-affinity glucose transporters expressed in oesophageal adenocarcinoma, respectively in decreasing order of magnitude of expression, GLUT8, GLUT4, GLUT3 and GLUT9. The highest Hscores in our series were obtained for GLUT8,

not GLUT1. Unlike GLUT1, under normal conditions, GLUT8 is localized intra-cellularly (Joost et al., 2001). Upon insulin or IGF-1 treatment, this transporter translocates to the plasma membrane, a movement that corresponds to an increase in glucose uptake mediated via the insulin-like growth factor (IGF)-1 receptor, not the insulin receptor. The insulin-like growth factor receptor and its agonist IGF-1 have been previously reported to be elevated in oesophageal neoplasia and to be related to poor outcome (Sohda et al., 2004; Iravani et al., 2003). Thus, hypothetically, high expression of GLUT8 as seen in our patient population may be related to IGF1/IGF1-R signalling in oesophageal adenocarcinoma.

Of interest, whereas GLUT 3 proved only moderately expressed in untreated, surgically removed tumours, significantly higher Hscores were found in those patients that first underwent radio-chemotherapy, as well as in patients that underwent primary surgery and had a previous history of alcohol or cigarette abuse. Under normal conditions, GLUT3 is responsible for neuronal glucose uptake and thus for the glucose uptake in the brain (Duelli and Kuschinsky, 2001). GLUT3 mRNA has, however, also been identified in a variety of human tissues and tumours (Younes et al., 1997a). In particular GLUT3 over-expression has been reported in non-small cell lung carcinoma, in colorectal cancer, pancreatic cancer and in squamous oesophageal carcinoma (Younes et al., 1997b). In both squamous cell carcinoma of the head and neck and non-small cell lung carcinoma GLUT3 over-expression was shown to be a poor prognosticator (Younes et al., 1997a; Baer et al., 2002). As compared to GLUT1, 4 and 8, GLUT3 is also capable of transporting galactose, mannose, xylose and maltose, in addition to glucose (Wood and Trayburn, 2003). Thus, hypothetically, tumour cells over-expressing GLUT3 may reflect the selection of clones that have a survival-advantage when compared to cells that do not over-express GLUT. Alternatively, the increased levels of GLUT-3 expression found following exposure to radiochemotherapy, cigarette or alcohol may reflect the activation of an innate defence mechanism.

Following trans-membrane transport, glucose is phosphorylated by hexokinases. Usually hexokinase II is the most prominent isoform associated with cancer. As opposed to hexokinase I, hexokinase II binds to the outer mitochondrial membrane of cancer cells and is directly coupled to ATP synthesis on the inner membrane (Pedersen et al., 2002). This provides high levels of glucose-6-phosphate that "jump start" the glycolytic pathway, ultimately leading to high levels of lactic acid in the presence of oxygen, i.e. the "Warburg effect" (Warburg, 1956). The receptor for hexokinase in the outer mitochondrial membrane is the protein named "VDAC" (Voltage dependent anion channel) (Azouly-Zohar et al., 2004). Binding of hexokinase II to VDAC also inhibits bax-induced cytochrome c release and thus apoptosis, providing a survival benefit to cancer cells (Pastorino and Hoek, 2003). In the series presented, hexokinase I proved to be the most prominent in oesophageal adenocarcinoma. Whether or not this also implies that hexokinase I contributes more to the overall glucose consumption by cancer cells is currently unclear. This issue warrants further exploration. Of interest, hexokinase II levels were significantly higher in those patients that were treated by radio-chemotherapy versus those patients that were treated by means of surgery. Again, hypothetically, this finding may reflect upregulation of a tumor cell defence mechanism or clonal selection of cells that have increased possibility for metastasis and that ultimately may lead to the death of the human host. Accordingly, adjuvant therapy targeting the hexokinase II enzyme in this patient population may prove worthwhile in the future to consider. A potential adjuvant agent in this regard is 3-bromopyruvate, which has been shown recently to eradicate advanced stage, PET positive hepato-cellular carcinomas in an animal model without apparent harm to the animals (Mathupala et al., 2006).

To conclude, oesophageal adenocarcinoma express a wide variety of GLUTs as well as all three hexokinase enzymes to a different extent. This expression pattern is significantly different in oesophageal adenocarcinoma that were previously treated by means of radiochemotherapy. Further studies relating FDG uptake to GLUT and hexokinase expression may help to better understand the contribution of GLUTs and hexokinases to overall tumor glucose consumption in patients suffering from oesophageal adenocarcinoma.

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