Fructose transporter GLUT5 expression in clear renal cell carcinoma

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Abstract. Renal cell carcinomas (RCC) can be subclassified for general purposes into clear cell, papillary cell, chromophobe cell carcinomas and oncocytomas. Other tumours such as collecting duct, medullary, mucinous tubular and spindle cell and associated with Xp 11.2 translocations/TFE 3 gene fusion, are much less common. There is also a residual group of unclassified cases. Previous studies have shown that RCC has high glycolytic rates, and expresses GLUT transporters, but no distinction has been made among the different subtypes of renal cell tumours and their grades of malignancy. In clear renal cell carcinoma (cRCC) glycogen levels increase, glycolysis is activated and gluconeogenesis is reduced. The clear cell subtype of RCC is characterized histologically by a distinctive pale, glassy cytoplasm and this appearance of cRCC is due to abnormalities in carbohydrate and lipid metabolism, and this abnormality results in glycogen and sterol storage. Several isoforms of glucose carriers (GLUTs) have been identified. We show here in a panel of 80 cRCC samples a significant correlation between isoform 5 (GLUT5) and many pathological parameters such as grade of differentiation, pelvis invasion and breaking capsule. GLUT5 expression also appears to associate more strongly with the clear cell RCC subtype. These data suggest a role for the GLUT5 isoform in fructose uptake that takes place in cRCC cells and which subsequently leads to the malignant RCC progression.

Introduction

Glucose is a major source of metabolic energy and virtually all animal cells possess a transporter system for glucose of the facilitative diffusion type.

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Glucose influx across the membrane cells is mediated by glucose transporters (GLUTs) which have at least 12 isoforms (1). The facilitative GLUTs use existing gradients in glucose (and other hexoses/polyols) concentration between the external and internal faces of a membrane to facilitate their translocation, thus ensuring a continuous supply of glucose to most tissues. These transporters are the products of distinct genes and exhibit considerable homology in their primary sequences but display a marked tissue-specific pattern of expression: the GLUT isoforms have different tissue distribution, function and developmental regulation. So far, different complementary DNAs (cDNAs) encoding these different species have been isolated (2-12), which have been named GLUT1 (expressed in all tissues and specially abundant in erythrocytes and brain), GLUT2 (present in liver, pancreatic islet ß cell, kidney and at the basolateral surface of the absorptive cells of the small intestine), GLUT3 (abundant in brain), GLUT4 (restricted to adipose, heart and skeletal tissues) and GLUT5 (expressed in small intestine, sperm cells and kidney) (Table I) (Fig. 1)

Laboratory *in vitro* and *in vivo* expression systems have demonstrated that these transporters not only function as glucose transporters but are also capable of transporting other sugars.

Among the GLUTs family members able to transport fructose, GLUT5 is the sole transporter specific for fructose with no ability to transport glucose or galactose (13).

The second major fructose transporter is GLUT2, a low-affinity transporter that is also capable of recognizing glucose and galactose. GLUT2 in a bidirectional manner is involved mainly in fructose uptake across the basolateral membrane of the intestinal and renal epithelial cells (14) after apical transport mediated by GLUT5, fructose is transported across the basolateral membrane by GLUT2 (Fig. 2). Kidney GLUT5 is therefore remarkably responsive to its substrate fructose. The response of GLUT5 is quite specific; GLUT2 expression is similar among fructose, glucose and non-metabolizable glucose analogs. Tissue-specific coordinated expression of glucose transporters could play an important role in the regulation of glucose uptake and metabolism under various nutritional and hormonal conditions. It has long been recognized that cancer cells have increased rates of glucose metabolism compared

Table I. Major sites of expression of the family glucose transporters (GLUT1-5).

Name	Tissue distribution	Role and important features	Refs.
GLUT1	Placenta, brain, blood-tissue barrier, muscle and adipose tissue, kidney	Basal glucose uptake in many cells kinetically asymmetric	(80-84)
GLUT2	Kidney (proximal tubule) liver, pancreatic ß-cell, small intestine (basolateral membranes)	High capacity, low affinity transporter important for glucose sensing in ß-cell transepithelial glucose and fructose transport	(4,80)
GLUT3	Brain, nerve cells, small intestine, kidney	Neural transporter role in small intestine unclear	(80,85-87)
GLUT4	Muscle and adipose tissue Heart	Expressed only in tissue that exhibit acute insulin-stimulated glucose transport translocates to plasma membrane in response to insulin	(88-100)
GLUT5	Jejunum (apical membranes), kidney, muscle and adipose tissue at low levels	Physiological role in fructose adsorption	(26-31,101)

with healthy cells (15-17). A variety of mechanisms have been proposed for the accelerated glucose use seen in growing tumours and in transformed and malignant cells: increased concentrations of hexokinase (18,19), decreased rates of glucose-6-phosphatase mediated dephosphorylation (20) and enhanced rates of glucose uptake have been noted. Moreover, tumour cells can apparently express glucose transporters that are not substantially expressed in the non-malignant tissue.

Renal cell carcinomas can be subclassified into clear cell carcinomas, papillary type I and II cell carcinomas, chromophobe cell carcinomas and oncocytomas.

The clear cell form of renal cell carcinoma is the most common type of renal tumours, and the cells are characterized histologicaly by a distinctive pale, glassy cytoplasm (21). It has been previously suggested (21) that the clear appearance of tumour cells results from some abnormality in the metabolism of carbohydrate and lipids and this abnormality results in glycogen and sterol storage.

It has been demonstrated that a series of characteristic changes occur in the carbohydrate metabolism of renal clear cell carcinomas: increase in glycogen synthesis, activation of glycolysis and gluconeogenesis reduction.

The above alterations of the carbohydrate metabolism within clear cell carcinomas are clearly distinct from those observed in papillary cell carcinoma, chromophobe cell carcinomas and oncocytomas (22).

The clear cell type of RCC has a typical golden colour due to the rich lipid content of its cells: cholesterol, neutral lipids and phospholipids are abundant (Fig. 3) (23). These accumulate as droplets in the cytoplasm of tumour cells due to deficient glycogenolysis and lipolysis associated with unresponsiveness of the tumour cell adenylate cyclase to glucagon. The lipid content is markedly similar to that of proximal convoluted tubules.

Microscopically, the cells of cRCC type are filed with lipids and cholesterol which are dissolved in usual histological preparations, creating a clear cytoplasm surrounded by a distinct cell membrane. These neutral lipids can be identified in unfixed material using Oil Red 0 and Sudan III and IV reaction. The phospholipids may be identified with the same stains. Glycogen can be identified using periodic acid-Schiff (PAS) or Best stain.

Previous studies demonstrated high glycolytic rates in renal clear cell carcinoma samples showing a series of characteristic changes occurring in the carbohydrate metabolism, however, the GLUT5 protein has not been implicated in this setting. The utility of fructose and function of GLUT5 in cRCC was shown to be uncertain. The GLUT5 is expressed in only a limited number of tissues seemingly capable of preferentially metabolizing fructose, and exist in two major categories of transcriptional and/or post-transcriptional regulation of GLUT5. In the apical membrane of polarized cells (e.g., kidney cells) GLUT5 is acutely and specifically regulated by its own substrate, whereas in the other tissues fructose seems to have no acute effect.

Because the GLUT5 transporter is commonly found in tissues that metabolize fructose, we hypothesized that cRCC may be capable of utilizing fructose as an energy substrate, and GLUT5 to collaborate in glycogen and cholesterol storage.

Material and methods

Patients and tumour samples. The clinical and pathological data of patients who were diagnosed with RCC and underwent surgery at the Department of Urology of Modelo Hospital, A Coruña, Spain, from 1996 to 2007 were reviewed. The study group consisted of 80 patients whose original pathological specimens were available for evaluation. The average age of the study population was 62 years being 66% male and 34% female. The histological study included 57 clear renal cell carcinomas, 6 papillary renal cell carcinomas, 15 chromophobe renal cell carcinomas and 2 samples with unknown histological type.

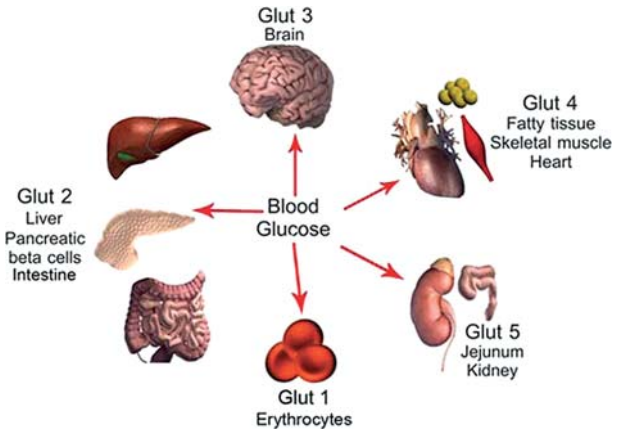

Figure 1. Many glucose transporters (GLUT1-5) are expressed in different human organs.

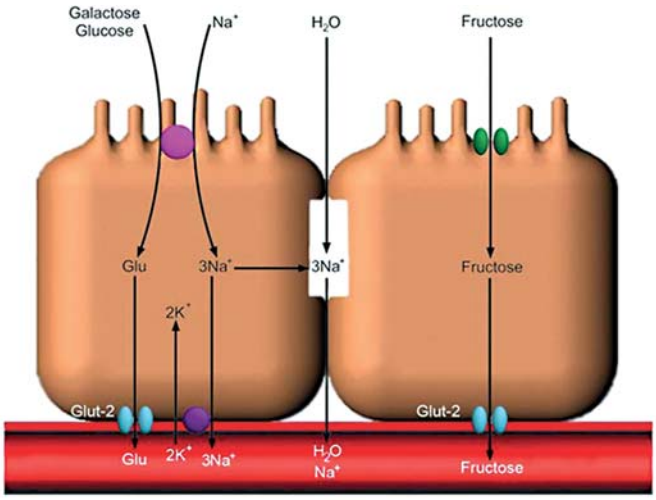

Figure 2. Among the GLUT family members able to transport fructose, GLUT5 is the sole transporter specific for fructose with no ability to transport glucose or galactose. The second major fructose transporter is GLUT2, a low-affinity transporter that is also capable of recognizing glucose and galactose.

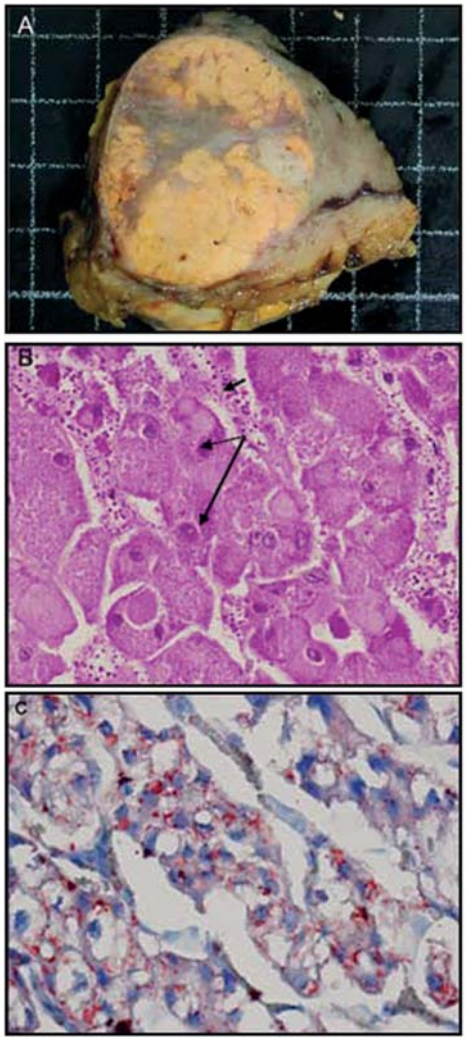

Figure 3. (A) The clear cell type of RCC is typically golden due to the rich glycogen and lipid content of its cells: cholesterol, neutral lipids and phospholipids. (B) Renal cell carcinoma stained with PAS, a dense granular deposition in the cytoplasm of cells (short arrow) and larger cells (long arrow) due to glycogen are visible (PAS x400). (C) Stained for cytoplasmic fatty deposits are seen as globules of different diameters. (Oil Red x400).

The Institutional Review Board of Modelo Hospital (A Coruña, Spain) approved the retrospective review of the medical records and the use of archived tumour specimens.

Tissue microarray generation. All archival tissue samples were routinely fixed in formalin and embedded in paraffin. Representative tissue areas were marked on standard haematoxylin and eosin sections, punched out of the paraffin block using 2.0-mm punch, and inserted in a recipient paraffin block, to produce a 6x8 array of 48 cases. In addition, one normal cerebellum tissue was inserted as a negative control. When it was possible triplicate cores per specimen were arrayed on a recipient paraffin block in order to decrease the error introduced by sampling and to minimize the impact of tissue during processing. Sections (4 μm) were cut from the completed array blocks and transferred to silanized glass slides.

Immunohistochemistry. The working dilution was determined using positive controls, as indicated in the literature. For GLUT1, oesophagus was used; for GLUT2, liver was used; for GLUT3, placenta was used, for GLUT4, heart was used and for GLUT5, small intestine was used. Additional sections, running in parallel but with omission of the primary antibody served as negative controls.

The tissue sections were deparaffinized by incubation in xylene and rehydrated in a graded series of ethanol and water solutions. The antigen was retrieved with 0.01 M citrate buffer (pH 6.0) by heating the samples in a microwave vacuum histoprocessor (2100 Retriever™, PickCell Laboratories) at a controlled final temperature of 121˚C for 15 min. The primary antibodies were diluted in Dako antibody diluent (DakoCytomation) with background-reducing components and were used at the followings dilutions: GLUT1-2 (1:50, Abcam), GLUT3 (1:25, Abcam), GLUT4-5 (1:250, Abcam). The primary antibodies were incubated at room temperature for 30 min and detected using the Dako EnVision system and diaminobenzidine according to the manufacturer's instructions.

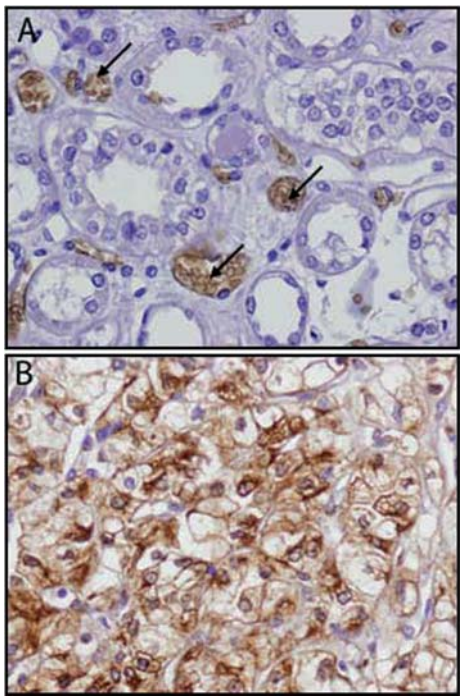

Figure 4. (A) GLUT1 immunostaining in RCC. The tubular epithelium, glomerulus and interstices are negative (x40). Intravascular erythrocytes are positive. (B) GLUT2 immunostaining in RCC. Moderate cytoplasmic positivity in 40% of tumour cells (x50).

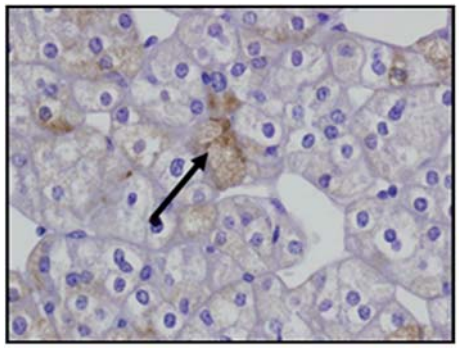

Figure 5. GLUT4 immunostaining in RCC. Light cytoplasmic positivity in 10% of tumour cells (x50).

Semi-quantification of antibody staining. The immunoreactivity score (IRS) was evaluated, following other groups, by multiplying the percentage of positive cells (PP %) and the staining intensity (SI). First, the PP % was scored as 0 for <1%, 1 for 1-24%, 2 for 25-49%, 3 for 50-74% and 4 for ≥75%. Second, the SI was scored as 1 for weak, 2 for medium and 3 for intense staining. Each slide was carefully examined in the area of the tumour that contained the greatest fraction of positively stained cancer cells.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). The statistical significance of differences found was evaluated at the 95% confidence level by non-parametric statistics, Mann-Whitney U and Kruskal-Wallis tests, p-values <0.05 were considered the cut-off point for significance. The statistical analyses were performed using commercially available software (SPSS 17.0 for Windows).

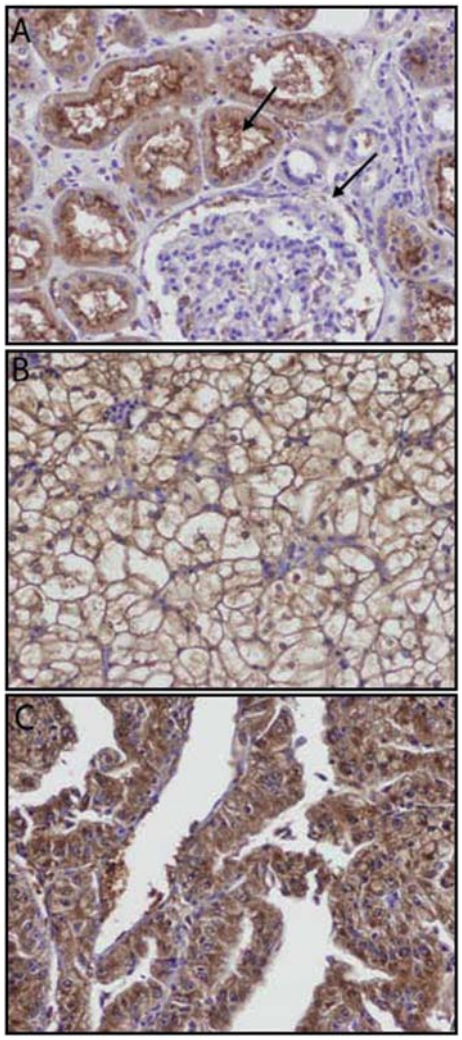

Figure 6. GLUT5 immunostaining. (A) Normal kidney with intense positivity in the apical pole of the tubular epithelium. The glomerulus, the interstices and the vascular endothelium are negative (x40). (B) Intense membrane positivity in most tumour cells (x50). (C) Intense cytoplasmic positivity in most tumour cells (x50).

Results

Tumour tissue samples from 80 RCC patients were analysed by immunohistochemistry for expression of different GLUT isoform (Figs. 4-6). Pathological characteristic of RCC patients are detailed on Table II.

GLUT1 showed membranous staining in red blood cells in the controls (Fig. 4A). Positive but weaker staining was also observed in 38 of 80 (47.5%) RCC samples where GLUT1 staining was observed in the plasmatic membrane and cytoplasm. Tubular epithelium, glomerulus and interstice were negative for GLUT1 staining. There was no correlation between GLUT1 staining and the pathological parameters considered for review as grade of differentiation, pelvis invasion and breaking capsule (Table II).

GLUT2 was detected as strong reaction in cell membrane in 58 of 80 (72.5%) RCC samples (Fig. 4B). Cytoplasm reaction could be observed with moderate intensity in 27.5%

Table II. Relationship between GLUT5 expression and pathological significance of RCC.

Pathological parameter	Patients no. n=80	GLUT5 range/ p-value
Differentiation degree (Fuhrman grade)		
Well	15	44.81/0.024
Moderate	45	
Poor	16	
Undifferentiated	4	
Pelvis invasion		
Yes	9	54.56/0.039
No	66	
Undetermined	5	
Breaking capsule		
Yes	12	54.75/0.019
No	64	
Undetermined	4	
Tumour depth		
1	63	56.63/0.437
2	4	
3	12	
4	1	
Histology type		
Clear cell	57	46.98/0.001
Papillary	6	
Chromophobe	15	
Undetermined	2	
Tumour localization		
Right	40	40.13/0.959
Left	39	
Undetermined	1	
Veins invasion		
Yes	9	63.00/0.312
No	67	
Undetermined	4	

Ranges are median.

of RCC samples. No significant correlation between the histological parameters studied and the expression of GLUT2 was seen (Table II).

GLUT3 was detected in 30 of 80 (37.6%) RCC samples showing weak cell membrane reaction and granular cytoplasmic staining. No significant correlation between the histological parameters studied and the expression of GLUT3 was seen (Table II).

GLUT4 was detected in a weak cytoplasmic pattern in 45 of 80 (56.3%) RCC samples (Fig. 5). Patients who were classified as T4 showed statistical significance of p=0.029, and higher GLUT4 expression than the others (Table II).

In normal kidney, we also observed GLUT4 stain on the apical pole of tubular epithelium. Vascular endothelium, glomerulus and interstice were negative. GLUT5 showed high intensity in the membrane and cytoplasm of tumoural cells in 46 of 80 (57.6%) RCC samples (Fig. 6B and C). A significant positive (p=0.024) correlation was found between moderately differentiate RCC tumour tissues and GLUT5. Patients who had pelvis invasion also showed significant (p=0.039) higher GLUT5 expression than the others. A significant positive (p=0.019). correlation was observed between GLUT5 expression and patients who had breaking capsule. Related to histological type we found that GLUT5 expression was significant higher in clear RCC (p=0.001) (Table II).

Discussion

In the kidney, *GLUT5* mRNA was shown abundant in the cytosol, and protein is present in the apical plasma membrane of S3 proximal tubule cells (24), where GLUT5 may potentially recapture fructose lost from glomerular filtration. Its expression is also inducible by the fructose diet (25) after the small intestine; the kidney expresses the most GLUT5 in human, rat and rabbit (26-31). Regulation of GLUT5 was first discovered in the intestine and testis, but also in the kidney, skeletal muscle, fat tissue and brain. Modest to significant levels of GLUT5 mRNA and/or protein have now been demonstrated in kidney, fat, skeletal muscle and brain (32-38). GLUT5 expression levels and fructose uptake rates are also significantly affected by diabetes, hypertension, obesity and inflammation (metabolic syndrome), and seem to be induced during carcinogenesis, particularly in the mammary glands (39).

The primary metabolic characteristic of malignant cells is an increased uptake of glucose and its anaerobic metabolism, and available evidence indicates that the mechanism by which cancer cells increase their ability to take up glucose involves the selective overexpression of glucose-transporters (40). It is currently accepted that the increase in glucose uptake by malignant cells is associated with the overexpression of GLUTs.

Overexpression of the facilitative glucose-transporter has been observed for a wide range of human cancers (41-58) with the degree of overexpression generally being inversely correlated with prognosis. The mechanisms by which GLUTs promote malignant cellular behaviour have focused on factors inducing its expression, such as local hypoxia (37), oncogenes such as Ras, Scr (15) or Myc (38).

In human renal cell carcinoma immunohistochemical staining GLUT1 was found in 73.3% of tumour specimens analyzed, and in the 84.6% of clear cell subtype (58), heterogeneous expression of GLUT1 was observed in tumour cell mass: some tumour cells were positive for GLUT1, while other cells were not (43). GLUT4 staining was not recognized in either tumour or normal tissues (58). Using RT-PCR in kidney tumours, it has been showed that histopathological types are characterized by specific patterns of GLUT expression (59).

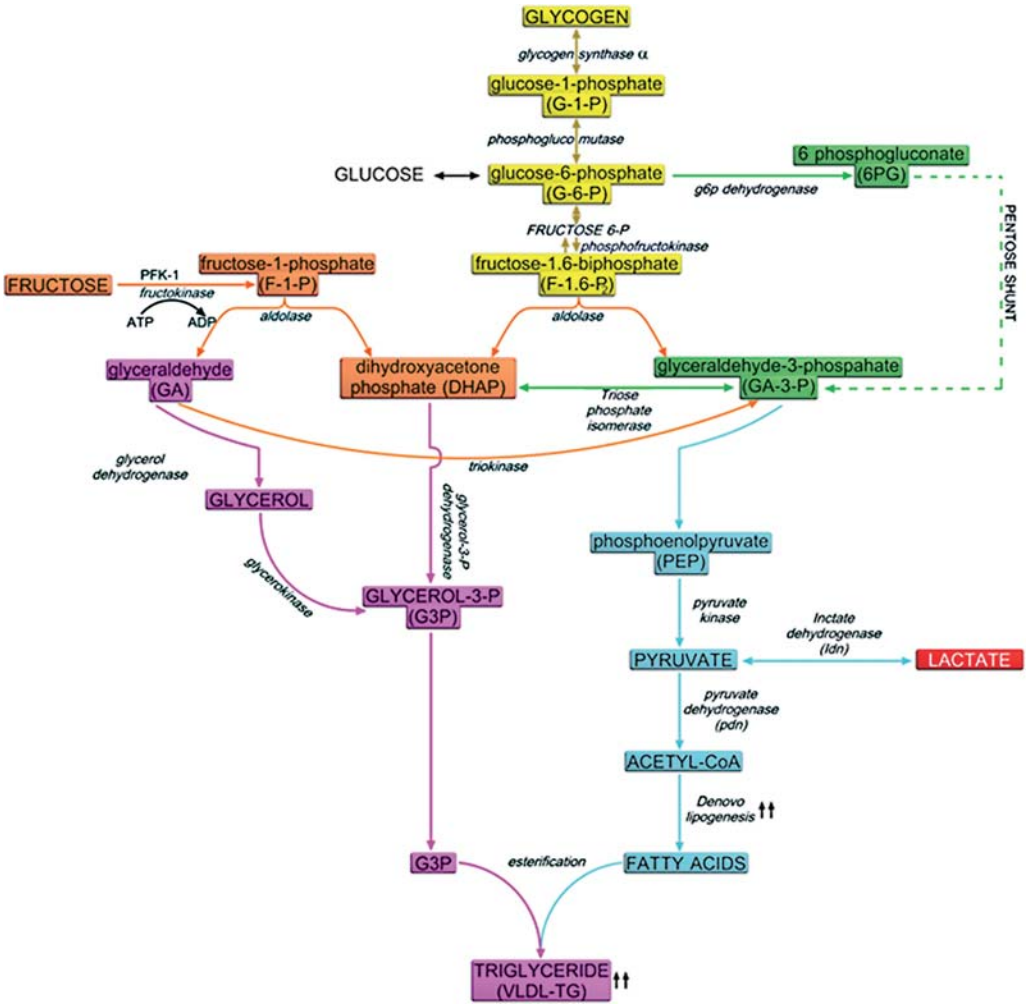

Figure 7. Steps in fructose metabolism.

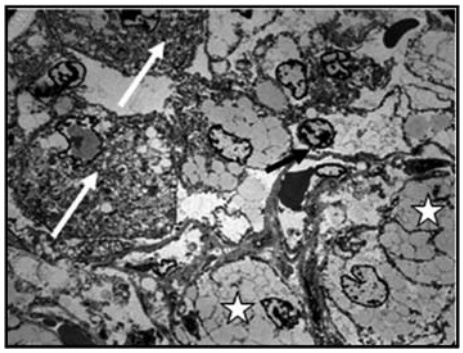

Figure 8. Surrounding a capillary (black arrow) there are tumour cells with abundant fat vacuoles (white stars). Other cells, probably in apoptosis (white arrow) contain lysosomes and grains of glycogen (x2800).

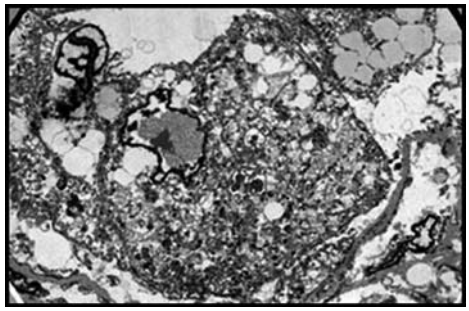

Figure 9. One tumour cell observed at higher magnification with electron microscopy showing clearly the grains of glycogen (x3500).

Fructose is now such an important component of human diets that increasing attention is being focused on the fructose transporter GLUT5. Fructose is transported passively across membranes by a member of the facilitative glucose transporter (GLUT) family, named GLUT5 (60-64) and it is the sole transporter specific for fructose with no ability to transport glucose or galactose. The low intracellular fructose concentration is possible because fructose is metabolized and significantly contributes to glycogenolysis in muscle or lipogenesis in adipocytes (63,65).

The products of fructose metabolism are glycogen and *de novo* lipogenesis of fatty acids and eventual synthesis of endogenous triglyceride can be divided into two main phases: the first phase is the synthesis of the trioses, DHAP and GA; the second phase is the subsequent metabolism of these trioses in either in the gluconeogenic pathway for glycogen

replenishment and/or the complete metabolism in the fructolytic pathway to pyruvate, which enters the Krebs cycles, is converted to citrate and subsequently directed toward *de novo* synthesis of the free fatty acid palmitate (66).

The first step in the metabolism of fructose is the phosphorylation of fructose by fructokinase and aldolase to yield fructose and respectively dihydroxyacetone (DHAP) glyceraldehyde (GA) and glyceraldehyde-3phosphate (GA-3-P) (Fig. 7).

The resultant GA then undergoes phosphorylation to GA-3-P. Increased concentrations of DHAP and GA-3-P drive the gluconeogenic pathway toward glucose-6-phospate (G-6-P), glucose-1-phosphate (G-1-P) and glycogen (Fig. 7). It appears that fructose is a better substrate for glycogen synthesis than glucose and that glycogen replenishment takes precedence over triglyceride synthesis.

Once organ glycogen is replenished, the intermediates of fructose metabolism are primarily directed toward triglyceride synthesis (Fig. 7-9).

In human adipocytes, a study demonstrated, hypoxia increases GLUT5 expression (9-fold) (64). Because hypoxia becomes more common during renal cell carcinoma (RCC) progression, it can be one of the factors leading to increases in GLUT5 expression in clear renal cell carcinoma (cRCC).

Along with GLUT5, the mRNA expression of key gluconeogenic enzymes, glucose-6-phosphatase (G-6-Pase) and fructose-1, 6-bisphosphatase (FBPase), increased significantly in clear renal cell carcinoma, suggesting a link between gluconeogenesis on the one hand and fructose transport as well as intracellular fructose on the other. FBPase activity is indirectly regulated by cAMP, which increases *in vivo* in the kidney epithelia exposed to fructose compared with those exposed to glucose. It has been demonstrated *in vivo* that cAMP modulates fructose transport induced by fructose without affecting GLUT5 mRNA abundance (65), whereas *in vitro*, cAMP affects GLUT5 mRNA expression levels and is involved in GLUT5 regulation in kidney epithelia (67-70).

GLUT5 mRNA and protein expression are affected by the development of tumours in certain organ systems. In general, oncogene-transformed cell that portray cancerous characteristics will also exhibit an increase in glucose transport by overexpression specifically sugar transporters like GLUT1 in breast cancer (71), colorectal (57) and like GLUT3 in lung cancer (72,73). Although GLUT5 is poorly expressed in normal kidney epithelial cells, the renal cell carcinoma tissue posses high amount of GLUT5 mRNA and protein and exhibit high rates of fructose transporter. This finding was confirmed a number of times in later studies. Screening of the GLUT5 in malignant vs. normal human tissues and cells showed that GLUT5 was highly overexpressed in 27% of cancerous tissues tested, including many type of human tumours (74).

We have also shown here, in a panel of 80 samples from RCC patients, significant higher expression of GLUT5 isoform was also found in RCC cells vs. normal kidney. In addition, GLUT5 staining appeared stronger in clear cell subtype than in others RCC histology types. These data suggest a main role for GLUT5 in glucose/fructose uptake in RCC tumour cells. In addition GLUT5 expression was correlated with clinicopathological features of advanced RCC.

In conclusion, by means of immunohistochemistry we have confirmed GLUT5 expression in clear renal cell carcinoma (cRCC), significantly in high Fuhrman degree, whereas GLUT1-4 expression was modest. The extensive expression of the glucose transporters, and the fact that in most of the cRCC overexpressing GLUT5 the rate of fructose uptake is exacerbated, indicate that fructose may be a preferred substrate providing energy required for the growth and proliferations of renal cell carcinoma of clear cell type. This increase of GLUT5 could indicate preferential utilization of fructose by renal cancer cells. The link between fructose and clear cell type of RCC was obvious. Interestingly, it was observed that cancer cells maintain a high rate of glycolysis even in presence of oxygen, a phenomenon called the Warburg effect (75,76).

One of the major regulatory steps in glycolysis involves conversion of fructose 6-phosphate to fructose-1, 6-bisphosphate by phosphofructokinase-1 (PFK-1). The activity of PFK-1 is allosterically controlled by fructose-2, 6-bisphosphate and the product of the enzymatic activity of a dual kinase/phosphatase family of enzymes (PFKFB1-4) that is also increased in a significant number of tumour types (77). Fructose is known to stimulate the intestinal expression of PFKFB1 (78), but it is not known whether fructose leads to increased levels of fructose 2, 6-bisphosphate. However, it is clear hat the rate of glycolysis can be stimulated by fructose because its entrance into glycolysis skips the two main regulatory enzymes (glucokinase and PFK-1) (79). Either the presence of high levels of GLUT5 protein leads to a greater use of fructose in neoplastic cells, in clear renal cell carcinoma or increased usage of fructose leads to a higher abundance of GLUT5 expression. The role of fructose in cRCC is clearly observed.

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