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ORIGINAL ARTICLE

U. Brinck · E. Eigenbrodt · M. Oehmke · S. Mazurek
G. Fischer

L- and M_2- pyruvate kinase expression in renal cell carcinomas and their metastases

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Abstract Using immunohistochemical and enzyme biochemical methods we investigated the expression of L- and M_2-pyruvate kinase (PK) in normal renal tissue, renal cell carcinomas (RCCs; of clear cell, chromophilic cell and mixed cell type) and RCC metastases. L-PK was expressed in the proximal tubules of normal renal tissue and, to a variable extent, in 23/25 primary RCCs, in 1 RCC recurrence and in 10 RCC metastases. Staining intensity and percentage of stained tissue did not correlate with tumour grade. One renal oncocytoma and all extrarenal malignancies examined lacked L-PK immunoreactivity. M_2-PK was mainly expressed in the distal tubules of the normal kidney and was found in all renal tumours as well as extrarenal malignancies. Quantitative biochemical investigations yielded a two- to seventeen-fold increase in PK activity in RCCs compared to the normal renal cortex taken from the same patient, whereas fructose-1,6-bisphosphatase and cytosolic glycerol-3-phosphate dehydrogenase activity was dramatically lower in RCCs. Otherwise, the activity of all other enzymes investigated (glucose-6-phosphate dehydrogenase, enolase and lactate dehydrogenase) was not significantly changed in the RCCs. The immunocytochemical results suggest that L-PK is a useful marker for RCC and its metastases, if acetone-fixed tissue is available. The quantitative changes of the concentration of PK and other enzymes in RCCs when compared with normal renal tissue probably reflect metabolic alterations related to tumour growth.

Key words L-pyruvate kinase · M_2-pyruvate kinase
Kidney neoplasms · Carbohydrate metabolism
Immunohistochemistry

U. Brinck (✉) · G. Fischer
Institute of Pathology, University of Göttingen,
Robert Koch Strasse 40, D-37075 Göttingen, Germany

E. Eigenbrodt · M. Oehmke · S. Mazurek
Institute of Biochemistry and Endocrinology,
University of Giessen, Frankfurter Strasse 100,
D-35392 Giessen, Germany

Introduction

Adequate treatment of individual cases cannot be given without accurate histological tumour classification by the pathologist. Organ-specific isoenzymes which remain within a tumour during the metastatic process are excellent indicators of organ specificity. Isoenzymes of carbohydrate metabolism, especially pyruvate kinase (PK) isoenzymes appear to be such indicators. PK isoenzymes (E.C. 2.7.1.40) have four subtypes: M_1, L, R and M_2. Investigations performed by our group have demonstrated that malignant tumours express PK type M_2 (M_2-PK) regardless of their histogenesis (Eigenbrodt et al. 1992). However, in the normal kidney, PK type L (L-PK) and M_2-PK are found in different cell types (Reinacher 1983).

In routine histopathology, the metastases of renal cell carcinomas (RCCs) are often erroneously diagnosed as primary tumours or as metastases originating from other sites. Since RCCs and their metastases are heterogeneous and, as their histochemical markers are not specific there is a need for new immunohistochemical tools to identify metastases. Our research group postulated that L-PK might be one such useful diagnostic marker for differentiating metastatic RCCs from other types of adenocarcinoma (Fischer et al. 1989).

The primary aim of this study was to test this hypothesis by examining the frequency of L-PK expression in primary and metastatic RCC. Secondarily, we chose to investigate the quantitative carbohydrate enzyme changes occurring during oncogenesis, including the immunohistochemical localization of PK isoenzymes and measurements of PK activity. In the light of the reports by several authors (Gerbracht et al. 1988; Eigenbrodt et al. 1992) that, in addition to switch PKs, continuous alterations of other carbohydrate enzymes occur during tumour formation, we also investigated whether such PK-related changes happen in RCC.

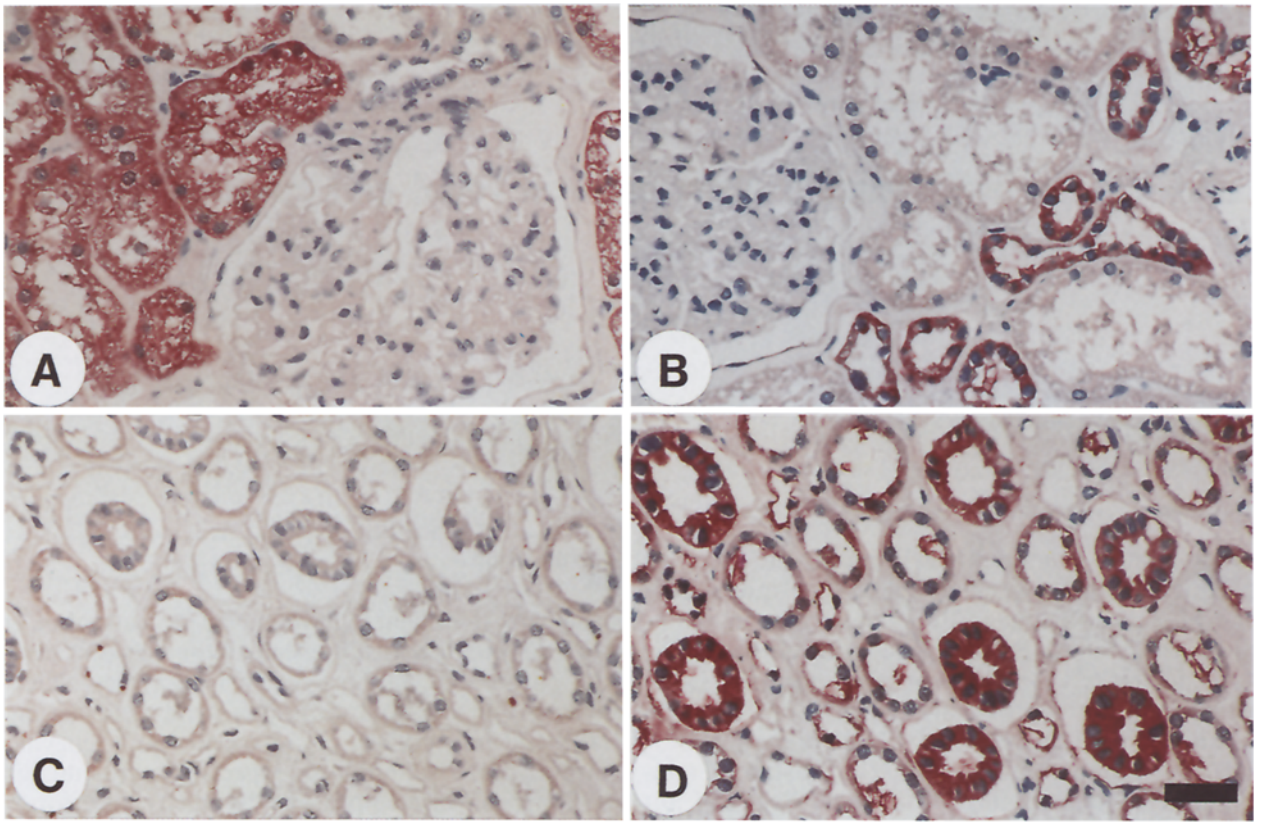

Fig. 1 Normal renal tissue. Immunohistochemical demonstration of L-PK in proximal renal tubules (**A**) and of M_2-PK in distal renal tubules (**B**), Henle's loops and collecting tubules of renal medulla (**D**). Note the lack of immunoreactivity for L-PK in the inner renal medulla (**C**). × 240. *Bar* = 42 μm

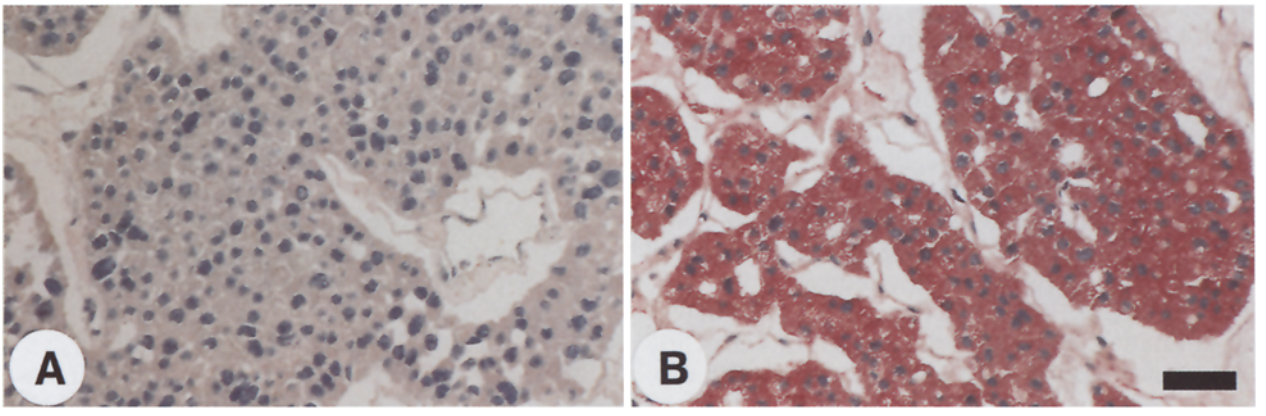

Fig. 5A, B Renal oncocytoma (**A**, **B**). Immunohistochemical demonstration of L-PK (**A**) and M_2-PK (**B**). × 240. *Bar* = 42 μm

Materials and methods

Surgical biopsies from 25 RCCs, 1 local recurrence of an RCC and 10 RCC metastases comprising tumours of clear cell type, chromophilic cell type and mixed cell type were examined. RCC metastases included 6 lymph node metastases, 1 metastasis in the small intestine, 1 metastasis in the thyroid gland and 2 bone metastases. Cytomorphological classification and grading was regional lymph node metastases of the clear cell carcinoma of the lung (3), liver metastasis of a gastric adenocarcinoma with clear performed according to Thoenes (Thoenes et al. 1986) using haematoxylin and eosin stained sections.

For control purposes, the following tumours were investigated: renal oncocytoma (1), well-differentiated squamous cell carcinoma of the renal pelvis (1), renal metastasis of a poorly differentiated squamous cell carcinoma of the lung (1), clear cell adenocarcinoma of the prostate (6), clear cell adenocarcinoma of the corpus uteri (5), clear cell adenocarcinoma of the cervix uteri (4), clear cell carcinoma of the ovary (2), clear cell carcinoma of the lung (1), cell appearance (1), papillary carcinoma of the thyroid gland (1), small cell anaplastic carcinoma of the lung (1) and liver metastasis of a small cell anaplastic carcinoma of the lung (1).

The tissue specimens were fixed in acetone and embedded in paraplast. Sections of 2 μm thickness were cut and examined immunohistochemically according to the alkaline-phosphatase-anti-

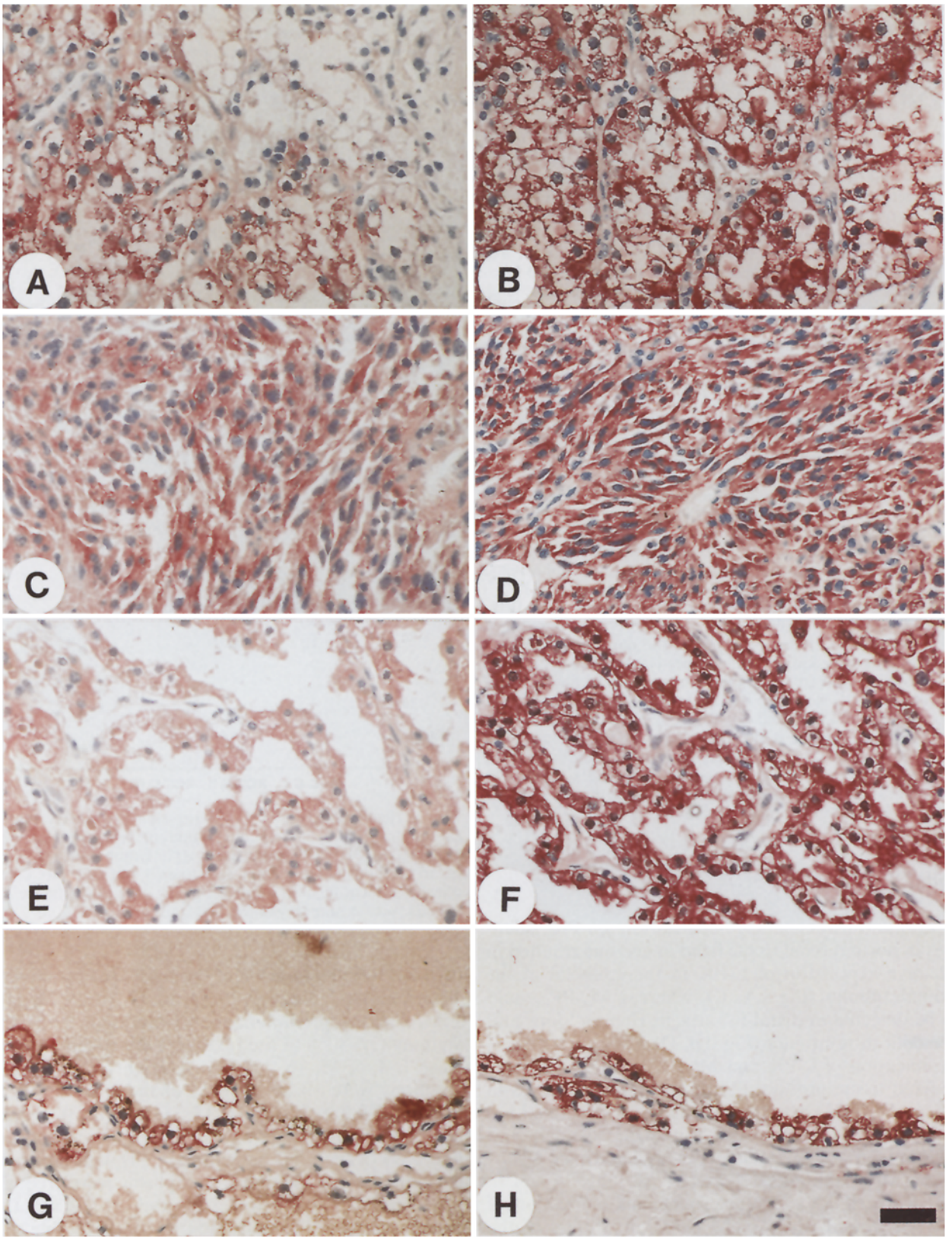

Fig. 2A–H Renal cell carcinomas: clear cell-compact (**A**, **B**), spindle-shaped cell-compact (**C**, **D**), chromophilic cell-tubulopapillary (**E**, **F**), clear cell-cystic (**G**, **H**). Immunohistochemical demonstration of L-PK (**A**, **C**, **E**, **G**) and M_2-PK (**B**, **D**, **F**, **H**) ×240. *Bar* = 42 μm

alkaline-phosphatase method (Cordell et al. 1984; Stein et al. 1985) for the presence of L-PK and M_2-PK. Ascitic fluid containing monoclonal mouse-anti-human L-PK of hybridoma cell line HME 2ED 7.1.1. (diluted 1:200 in TRIS buffer) as well as ascitic fluid containing monoclonal mouse-anti-human M_2-PK of hybridoma cell line HRA 10 DF 4 B7 AG (1:1 in TRIS buffer) served as primary antibodies (provided by ScheboTech, Wettenberg, FRG).

To control the specificity of the antibody binding to PK isoenzymes, L- and M_2-PK were localized in normal renal tissue taken from resected kidney of the same patient, both adjacent to and as distant as possible from the site of the primary tumour. Acetone fixation of the tissue was chosen troughout the study because comparative immunohistochemical reactions against L-PK and M_2-PK on 3.6% formaldehyde-fixed renal tissue resulted in negative staining. For the negative control of the immunohistochemical reaction, ascitic fluid was replaced by the supernatant fluid of the culture of myeloma cell line SP2/0, whereby all control reactions both in the tumour tissue and in normal renal tissue were negative.

For semi-quantitative evaluation of the immunohistochemical reactions, the staining intensity was grouped into four categories (negative, weakly positive, moderately positive, strongly positive). The staining intensity of normal renal tissue was categorized as "strongly positive". The PK-positive areas and the intensities were quantified by determining the area integral with the Leitz Elas 200 system. In each case, three sections from three different tissue blocks were investigated planimetrically and from these, the mean values were calculated.

Enzymatic activities of PK, fructose-1,6-bisphosphatase, glucose-6-phosphate dehydrogenase, cytosolic glycerol-3-phosphate dehydrogenase, enolase and lactate dehydrogenase (E.C. 1.1.1.27) were measured according to Gerbracht (Gerbracht et al. 1988; Hugo et al. 1992) in 6 cases of RCC clear cell type, comprising 2 grade I and 4 grade II tumours and in the normal renal cortex takem from the same kidney of the respective patients, but located as distant as possible from the site of the primary tumour. Five of these cases of RCC were also studied immunohistochemically for the expression of L-PK and M_2-PK. Enzymatic activities were related to tissue weight (U/g). Differences in enzyme activities between normal renal cortex and RCCs were tested for statistical significance with the t-test for connected probes ($P \leq 0.05$). For statistical evaluation variables were converted to their logarithms because no normal distribution was found.

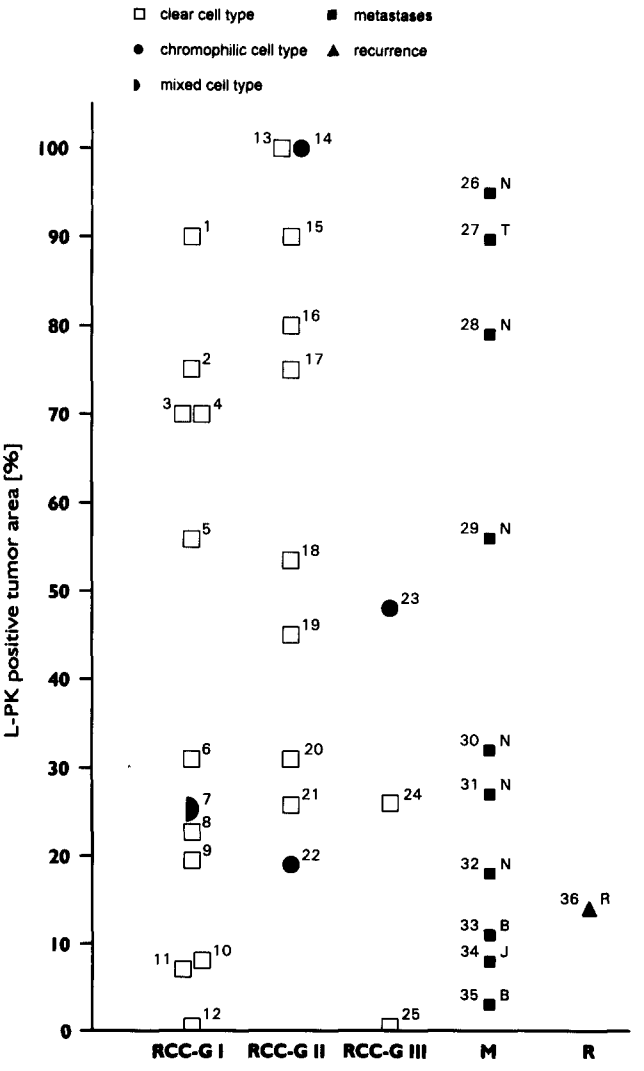

Fig. 3 Heterogeneity of L-PK expression in primary renal cell carcinomas (RCC) in relation to tumour cell type and tumour grade (*G*) and in RCC metastases (*M*) in lymph node (*N*), thyroid gland (*T*), bone (*B*) and small intestine (*I*) and in an RCC recurrence (*R*). Identification number of each case in the figure corresponds to case numbers in the text and in Table 1

Results

The immunohistochemical localization of PK isoenzymes in normal renal tissue fixed in acetone resulted in a selective expression of L-PK in the epithelia of the proximal tubules (Fig. 1A, C), whereas M_2-PK could only be detected in distal tubules, in Henle's loops and in the collecting tubules (Fig. 1B, D).

Application of L-PK and M_2-PK antibodies to acetone-fixed tissue produced strong staining reactions in L-PK and M_2-PK expressing cells in normal renal tissue. In contrast, immunoreactions against L-PK and M_2-PK on 3.6% formaldehyde-fixed renal tissue resulted in negative staining.

In clear cell RCCs, a heterogeneous expression of L-PK was found in 20 of 22 cases, regardless of the growth pattern (compact, Fig. 2A; tubulopapillary; tubulocystic, Fig. 2G) or the degree of malignancy, whereby all degrees of intensity of the immunohistochemical reaction were observed side-by-side (Fig. 2A). In the positive tumour areas, there was a predominance of weak staining (6–83% of the total area examined) in 14 cases (case 2, 3, 6, 8, 9, 10, 11, 13, 17, 19, 20, 21, 24, 31; Fig. 3) and a predominance of moderate and strong staining (29–80% of the total area examined) in 6 cases (case 1, 4, 5, 15, 16, 18; Fig. 3). Highly positive L-PK reactions were observed in 11 of the 20 positive cases (case 1, 3, 4, 5, 8, 13, 15, 16, 17, 18, 19; Fig. 3) where the area with highly positive L-PK reactions was between 2 and 50% of the total tissue area. Cells with moderately eosinophilic and granular cytoplasm were frequently observed in clear cell RCCs of grade II and III. L-PK expression within these tumours was detectable to the same extent in typical clear cells with "empty" cytoplasm as in moderately eosinophilic/granular cells, classified by Thoenes et al. (1986) as cells with clear cell eosinophilia. The L-PK immunoreactivity of tumours with extensive clear cell eosinophilia (case 15, 16, 20, 24,

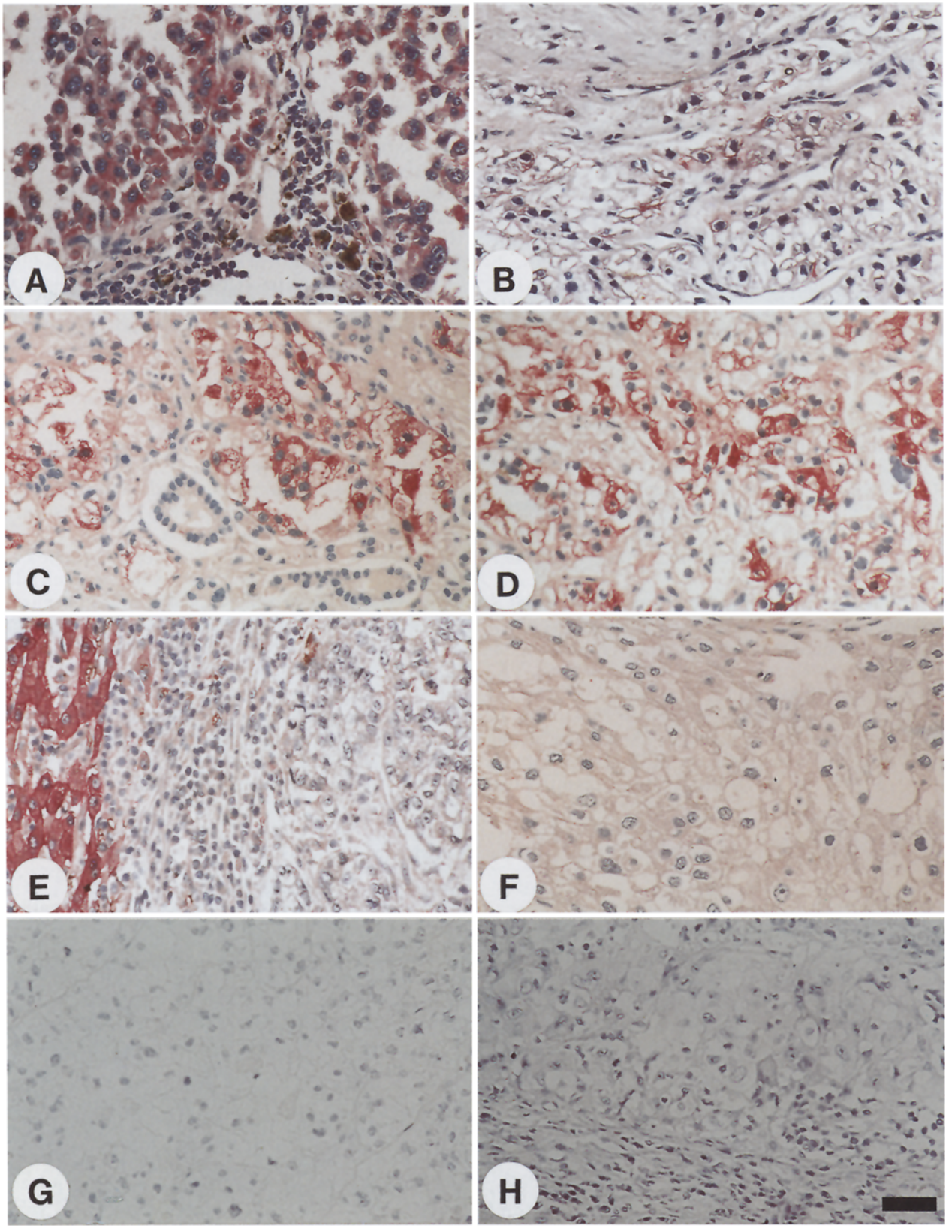

Fig. 4A–H Metastases of renal cell carcinomas in lymph node (**A**), small intestine (**B**), thyroid gland (**C**) and bone (**D**, **F**), and metastasis of gastric adenocarcinoma in liver (**E**). Clear cell adenocarcinoma of the prostate (**F**). Clear cell adenocarcinoma of the corpus uteri (**G**). Lymph node metastasis of clear cell carcinoma of the lung (**H**). Immunohistochemical demonstration of L-PK. × 240. *Bar* = 42 μm

25; Fig. 3) did not differ from that of typical clear cell tumours with "empty" cytoplasm.

Chromophilic cell RCCs also (3 cases; Fig. 2E) exhibited L-PK expression ranging from negative to strong staining reactions, regardless of the degree of malignancy. Positive areas comprised 19% (case 22; Fig. 3), 48% (case 23; Fig. 3) and 100% (case 14; Fig. 3). Here, weak staining intensity was common (26%, case 22; 61%, case 23; and 91%, case 14, of the L-PK-positive areas).

In an RCC of mixed type (case 7; Fig. 3) L-PK was detected in 25% of the tumour area. The staining reaction was strong in 4%, moderate in 5% and weak in 16% of the tumour area. In a local recurrence of the same tumour 5 years after nephrectomy (case 36; Fig. 3), the L-PK-positive area was reduced to 14% of the area of the local recurrence; thus the staining intensity was stronger in the recurrent lesion.

In spindle-shaped and pleomorphic tissue areas of chromophilic and mixed cell RCCs, there was a predominance of negative and weakly positive L-PK staining. Only small tissue areas and disseminated cells reacted moderately or in a highly positive way in these tumours (Fig. 2C). Both of the two L-PK negative RCCs were of the clear cell type (compact, grade I and compact, grade III).

In all 6 RCC lymph node metastases, positive L-PK expression was detected in between 18 and 95% of the tumour area. A preponderance of moderate staining intensity was observed in 4 cases (60–80% of the L-PK-positive tumour area; case 29, 30, 31, 32; Fig. 3) and of weak but significant staining in 2 cases (70% of the L-PK-positive tumour area; case 26, 28; Fig. 3). Strong staining intensity was observed in 3 of the 6 cases (Fig. 4A; 2–20% of the L-PK-positive tumour area; case 26, 30, 31; Fig. 3). The RCC metastasis in the thyroid gland showed a clearly positive L-PK reaction with 90% of the tumour area reacting positively (Fig. 4C). In these tumour areas 88% were strongly stained, 10% moderately and only 2% weakly. L-PK was not detectable in normal thyroid gland tissue (Fig. 4C). Only 11% of the total tumour area in an RCC bone metastasis (case 33; Fig. 3) was L-PK-positive; 53% of L-PK-positive areas showed a strong immunohistochemical reaction, 27% a moderate, and 20% a weak one (Fig. 4D). A second case of RCC bone metastasis (case 35; Fig. 3) had a positive anti-L-PK reaction in only 3% of the tumour area (moderate to strong staining reaction). The RCC metastasis in the wall of the small intestine was found to express L-PK in only 8% of the tumour area (weak staining in 62% and medium staining in 38% of the positive tumour area, Fig. 4B).

M_2-PK was detectable in 100% of the tumour cells of all primary RCCs as well as in the thyroid gland and bone metastases. The majority of these tumour cells were strongly stained (Fig. 2B, D, F, H).

The renal oncocytoma exhibited a completely negative reaction to L-PK, whereas M_2-PK expression was strong in all tumour cells (Fig. 5A, B).

A positive L-PK reaction was not detectable in any of the non-renal cell carcinomas, i.e. the clear cell adenocarcinomas of the prostate (Fig. 4F), of the corpus uteri (Fig. 4G) and the cervix uteri, the clear cell carcinomas of the ovary, the lung and its lymph node metastases (Fig. 4H), the papillary carcinoma of the thyroid gland, the squamous cell carcinoma of the renal pelvis, the small cell anaplastic carcinoma of the lung, the renal metastasis of a squamous cell carcinoma of the lung, the liver metastasis of a gastric adenocarcinoma (Fig. 4E) and the liver metastasis of a small cell carcinoma of the

Table 1 Enzyme activity (U/g) in 6 RCCs (clear cell-compact) and 6 normal renal cortices (control) of the same patients

Case number[a]	Tissue sample	Fructose-1,6-bisphosphatase	Glucose-6-phosphate dehydrogenase	Cytosolic glycerol-3-phosphate dehydrogenase	Enolase	Pyruvate kinase	Lactate dehydrogenase
37[e]	N[b]	3.1	6.92	51.4	72.9	99.9	558.9
37	T[b]	0.6	4.59	1.6	46.2	176.5	256.9
4	N	0.7	3.24	12.8	58.5	78.4	291.2
4	T	1.4	14.13	9.4	85.8	812.1	848.9
17	N	1.9	3.91	33.0	53.7	76.6	312.4
17	T	0.8	5.15	2.9	120.1	692.2	400.5
16	N	1.1	4.17	12.7	48.2	31.9	251.5
16	T	0.3	5.76	9.0	112.5	536.9	752.6
13	N	1.4	3.88	13.7	38.8	28.6	177.1
13	T	0.5	8.49	2.2	60.2	355.0	268.2
15	N	2.4	3.29	31.4	44.4	43.9	314.3
15	T	0.2	1.37	1.0	32.2	226.8	265.7
Mean	N	1.8	4.2	25.8	52.8	59.9	317.6
Mean	T	0.6[c]	6.6	4.4[c]	76.2	466.6[d]	465.5

[a] Corresponds to case numbers in Fig. 3
[b] N = normal renal cortex
T = tumor
[c] Statistically significant difference between N and T ($P < 0.05$)
[d] Statistically significant difference between N and T ($P < 0.01$)
[e] Case not investigated immunohistochemically

lung. In the case of liver metastases of gastric and lung carcinoma, positive immunohistochemical L-PK reaction in hepatocytes served as a useful internal positive control of the immunohistochemical reaction, even if the liver parenchyma had been damaged by the metastatic tumour. However, the well-differentiated squamous cell carcinoma of the renal pelvis was strongly M_2-PK-positive. The thyroid gland carcinoma showed a moderate reaction and the renal metastasis from a squamous cell carcinoma of the lung exhibited a heterogeneous, partly moderate, partly strong reaction to M_2-PK.

The enzymatic activity of 6 clear cell type tumours (one grade one, and five grade two) are listed in Table 1. PK activity in cancer tissue was clearly elevated compared to normal renal cortex ($P<0.01$), whereby the enzyme activity ratio (tumour/renal cortex) varied between 1.8 (case 37, Table 1) and 17 (case 16, Table 1). Strong differences in PK activity amongst the RCC cases did not coincide with relevant differences in immunohistochemically detectable L-PK or M_2-PK expression in terms of immunoreactive tumour area (Table 1, Fig. 3). However, in morphologically normal renal cortex there was also some degree of variation in PK activity among the different patients, ranging from 28 U/g (case 13, Table 1) to 99,9 U/g (case 37, Table 1). Fructose-1,6-bisphosphatase and cytosolic glycerol-3-phosphate dehydrogenase activity in RCC was reduced in comparison to the enzyme activity in normal renal cortex ($P<0.05$). The activity of glucose-6-phosphate dehydrogenase, enolase and lactate dehydrogenase did not show significant differences between RCCs and normal renal cortex.

Discussion

The main finding of this study is that RCCs and their metastases can be distinguished from other malignancies by their common expression of L-PK. This L-PK expression, however, is only unequivocally detectable in tissue properly fixed in acetone.

Immunohistochemical detection of L-PK in metastatic RCC is of diagnostic relevance if the diagnosis proves difficult on the basis of morphology alone. Accurate diagnosis is a prerequisite for adequate therapy of metastatic RCC and is of practical importance in the light of the new therapeutic regimens which yield reproducible anti-tumour effects and other therapeutic measures being tested in clinical studies (Quesada 1989; Rugarli 1989; Debruyne et al. 1990; Haas et al. 1993).

Earlier studies have also described the detection of specific antigens and glycoproteins of proximal renal tubules in RCCs and RCC metastases (Vesella et al. 1985; Oosterwijk et al. 1986). Most of these markers were detectable in cases of non-RCC and other markers were only occasionally found in metastases, limiting their practical application. Nevertheless, the search for antigens of practical diagnostic utility as immunohistochemically detectable differentiation products in metastatic RCC has recently borne fruit (Yoshida and Imam 1989; Ang et al. 1991). GP 200 was demonstrated in 84% of 58 metastatic RCCs and prealbumin was found in all of the 9 investigated metastatic RCC. Both antigens have the practical advantage of being detectable in formaldehyde-fixed tissue, but they lack specificity because they can also be detected in other tumours of non-renal origin. However, it is still essential that a conclusive comparison of the expression of L-PK, GP 200 and prealbumin in RCC metastases be undertaken.

The fact that L-PK, a proximal renal tubule differentiation molecule, is expressed in clear cell and chromophilic RCC provides further evidence that the majority of clear cell and chromophilic RCCs in humans may arise from the proximal tubules as previously proposed by other authors (Störkel and Jakobi 1989; Fischer et al. 1991).

Oncocytomas both in humans and in the rat originate from the collecting duct system (Nogueira and Bannasch 1988; Störkel and Jakobi 1989). The lack of immunohistochemically detectable L-PK in a human oncocytoma in this study is consistent with the concept that this tumour group does not derive from proximal renal tubules. This finding encourages further systematic studies to answer the question whether L-PK may be useful as an immunohistochemical marker for the differential diagnosis of oncocytoma and renal cell carcinoma. This would involve distinguishing between eosinophilic RCC (including tumours with cellular characteristics similar to oncocytes) and the diffuse eosinophilic/granular variant of clear cell RCC on the one hand and oncocytomas on the other, allowing the diagnostic decision between benign (oncocytic) and malignant (RCC) renal tumour entities. Furthermore, future investigations will show whether L-PK is useful in the diagnosis of chromophobic and collecting-duct carcinoma. The expected lack of L-PK expression in these two tumour entities points to their non-proximal renal tubule histogenesis. Other authors also suspect, with varying degrees of certainty, that these tumour entities are histogenetically related to the collecting tubules (Thoenes et al. 1989; Störkel et al. 1989; Ortmann et al. 1991; Störkel 1993).

All clear and chromophilic RCCs investigated express M_2-PK homogeneously, whereas most of them express L-PK heterogeneously. L-PK, but not M_2-PK, is detectable in proximal renal tubules, the probable origin of clear and chromophilic RCCs (Störkel and Jakobi 1989; Fischer et al. 1991). Our investigations, therefore, suggest that there is an incomplete shift from L-PK to M_2-PK during RCCs tumorigenesis. This shift from L-PK to M_2-PK indicates a change in glucose metabolism from energy production to the essential performance of synthesis (Eigenbrodt et al. 1985).

Our data clearly show that immunohistochemistry provides precise information on the individual distribution of isoenzymes in tumour and normal tissue and on

the origin of the metastatic tissue. Enzymatic measurements gave an exact quantification of PK activity, but they cannot discriminate between L-PK and M_2-PK. Our investigations have demonstrated that a degree of variability of PK activity exists between the renal cortex tissue specimens of different patients. Since these renal cortex tissue specimens showed no histopathological changes, differences in PK activities might be due to metabolic conditions, as has been shown after potassium depletion in rats (Aithal et al. 1980).

Comparison of normal kidney cortex with RCC of the same patient reveals that all tumour tested have much higher PK activities than normal tissue. The result is consistent with reports by other investigators (Steinberg et al. 1992; Eigenbrodt et al. 1992) who demonstrated that PK activity measured under maximal conditions is increased in all malignant tumours. Increased activity of pyruvate kinase was also recently demonstrated by Ahn et al. (1992) in N-nitrosomorpholine-induced clear and acidophilic renal cell tumours of the rat. The alteration in PK isoenzyme expression supports the concept that fundamental shifts in carbohydrate metabolism are essential for tumour formation in the kidney (Ahn et al. 1992).

In rat tumours (Reinacher and Eigenbrodt 1981) and in human cell lines (Board et al. 1990), there is a correlation between the metastatic potential and the maximal activity of PK (Eigenbrodt et al. 1992). Whether this holds true for human RCCs must be shown by future investigations. In agreement with other investigations (Haimoto et al. 1986; Eigenbrodt et al. 1992; Steinberg et al. 1992), there is no general increase in glucose-6-phosphate dehydrogenase or enolase activity in RCCs, but there is a constant decrease in fructose-1,6-bisphosphatase. This is the first investigation to show that the constant decrease in cytosolic glycerol-3-phosphate dehydrogenase found in rat liver tumours is also present in human RCCs (Gerbracht et al. 1988; Eigenbrodt et al. 1992). These enzymatic alterations are necessary for the expansion of the phosphometabolite pools between phosphoenol pyruvate and glucose-6-phosphate (Eigenbrodt et al. 1992; Hugo et al. 1992; Mazurek et al. 1993). These phosphometabolites are essential for synthetic processes and for the regulation of the protein kinases which play a major role in cell proliferation. The high glycolytic activity is necessary for the tumour cell if vascularization is bad, and in order for tumour cells to migrate (Beckner et al. 1990).

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