Decrease of mitochondrial DNA content and energy metabolism in renal cell carcinoma

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To elucidate the relationship between tumorgenesis and the mitochondrial energy metabolism in renal neoplasms, we studied three individual enzyme activities of the oxidative phosphorylation, two components of the Krebs cycle and the mitochondrial DNA content of renal carcinomas including 29 conventional, five papillary, two unclassified carcinomas with sarcomatoid features and one collecting duct carcinoma. A significant reduction of all mitochondrial enzyme activities including complex V, as well as of the mitochondrial DNA content was detected in 34 of 37 renal carcinoma tissues as compared with control kidney. Mitochondrial enzyme activities and mitochondrial DNA levels were not statistically different between the conventional, papillary and unclassified sarcomatoid type of renal carcinoma and did not correlate with tumour grade, metastasis, ploidy and proliferative activity as determined by Ki-67 staining. Taken together, our data indicate that a co-ordinated down-regulation of all components necessary for mitochondrial energy metabolism occurs in most renal carcinomas as an early event in carcinoma formation, which does not change with progression of the disease.

Introduction
Renal tumours are heterogeneous in origin and can be subdivided into the benign renal oncocytoma, papillary renal adenoma, metanephric adenoma and the following malignant renal carcinomas: conventional (clear cell), papillary (chromopholic), chromophobe, collecting duct and unclassified (1,2). Sarcomatoid change has been found to arise in all types of renal carcinoma not being a type per se, but rather an indication of tumour progression associated with poor prognosis (1). Conventional renal carcinoma, the most common renal malignancy (70%), as well as papillary and chromophobe renal carcinoma arise in the epithelium of the proximal renal tube. Collecting duct renal carcinomas arise from the medullary collecting ducts (3).

Studies by Warburg over five decades ago demonstrated that the vast majority of human and animal tumours display a high rate of glycolysis under aerobic conditions (4). Human solid tumours endure profound hypoxia, which indicates that adaptation to hypoxic conditions is a crucial step in tumour progression. The anaerobic use of glucose as an energy source through glycolysis is therefore a feature common to several solid tumours, in turn leading to a lesser dependence on the mitochondria for oxidative phosphorylation (5,6).

There is increasing evidence that mitochondrial mutations and/or functional abnormalities are associated with various neoplasms, although it is not clear whether mitochondrial lesions are contributing factors in carcinogenesis or whether they simply arise as part of secondary effects in cancer development (7-10). In many solid tumours the changes of the energy metabolism are associated with a reduction of the mitochondrial DNA content and the activity of enzymes of oxidative phosphorylation (OXPHOS) and are frequently related to the aggressiveness of the tumours including hepatocellular and renal carcinoma (11-13).

To elucidate if there is a link between malignancy and changes of the mitochondrial energy metabolism in renal carcinomas we examined nephrectomy specimens from 37 patients. In each case, tissue removed from the corresponding healthy part of the kidney served as matched control for the renal tumour tissue. For each set of paired specimens we investigated the relationship of the activity of OXPHOS and Krebs cycle enzymes and the amount of mitochondrial DNA in correlation to cell proliferation, ploidy, tumour grade and histological type of renal carcinoma.

Materials and methods

Patients
During a period of 2 years (from January 2001 to November 2002) fresh tumour and the corresponding healthy cortex tissues were obtained by nephrectomy at the Department of Urology, Salzburg. The tissues were snap frozen in N2 within 30 min of surgery and stored at −80°C. The tumour classification was performed according to Störkel et al. (1). We analysed 31 conventional renal carcinomas (n = 17 grade 1 + 2, n = 14 grade 3 + 4) including two unclassified carcinomas with sarcomatoid features, five papillary and one collecting duct carcinoma. The average age of all 37 patients was 64 ± 12 years with a range of 36–85 years, 25 of them were male.

Quantitative Southern blot analysis
The relative amount of mitochondrial DNA was determined by Southern blot analysis by correlating the mitochondrial to the 18S nuclear DNA signal. Total cellular DNA was isolated from frozen renal tissues according to standard procedures. Southern blot analysis was carried out as described previously (14). The same mixture of mitochondrial and nuclear probes was used in each experiment. In order to determine the intensity of the signals, densitometry on autoradiographs was performed. The relative amount of mitochondrial DNA was expressed as the ratio of the signal of the mitochondrial probe to that of the nuclear probe (m/r ratio).

Ploidy
Fresh renal carcinoma and control tissues were touched onto poly-l-lysine slides, Feulgen stained and DNA cytometry was performed with the ACAS.
DNA system (Ahrens ACAS, Bargteheide, Germany) (15,16). To determine ploidy, 200 renal cells of each specimen were measured. Leukocytes and granulocytes were used as reference cells.

Immunohistochemistry
Immunofluorescence with a monoclonal antibody directed against human mitochondrial porin (A-21317; Molecular Probes, Eugene, OR) and cytochrome c oxidase (COX) subunit I (A-6403; Molecular Probes) was performed according to the manufacturer’s instructions. In the present study we used formalin-fixed, paraffin-embedded material (2 μm) from renal carcinomas and the corresponding control material. After deparaffinization in xylene and gradient alcohol, treatment with 0.01 M sodium citrate for 15 min at 95°C followed. The slides were incubated for 2 h with the primary antibody (porin, diluted 1:30 in PBS; COX subunit I, diluted 1:30 in PBS). After washing the sections three times for 5 min in PBS, slides were incubated for 30 min with a FITC-labelled goat anti-mouse secondary antibody (AB 124 F; Chemicon, Temecula, CA) diluted 1:100 in PBS.

Sample preparation for enzyme measurements and western blot analysis
Renal tumor and control tissues (20–100 mg) were homogenized with a tissue disintegrator (Ultraturrax, IKA, Staufen, Germany) in extraction buffer (20 mM Tris–HCl, pH 7.6, 250 mM sucrose, 40 mM KCl, 2 mM EGTA) and finally homogenized with a motor-driven Teflon-glasm homogenizer (Potter S, Braun, Melsungen, Germany). The homogenate was centrifuged at 600 g for 10 min at 4°C. The supernatant (600 g homogenate) containing the mitochondrial fraction was used for measurement of enzyme activities and western blot analysis.

Enzyme measurements
The following enzyme activities were determined: citrate synthase (17), COX (18), complex II (19) with modifications (20) and isocitrate dehydrogenase (21) with the following modifications to the assay buffer: 50 mM HEPES pH 7.6, 2 mM MnCl₂, 4 mM ta-isocitrate and 0.1 mM NADP (nicotinamide adenine dinucleotidephosphate). Oligomycin and aurovertin-sensitive ATPase activity of complex V was determined using buffers described by Rustin et al. (19), but by sonifying the whole reaction mixture for 10 s with an ultra-sonifier (Bio cell disruptor 250, Branson, Vienna, Austria) at the lowest energy output (22). The concentration of oligomycin was 3 μM and of aurovertin B 60 μM. All spectrophotometric measurements (Uvicon 922, Kontron, Milano, Italy) were performed at 37°C.

Western blot analysis
After separation of the 600 g homogenate (18 μg protein/lane) on 5–20% polyacrylamide gradient gels, western blot analysis was performed according to Berger et al. (20).

The following antibodies were used: mouse monoclonal antibodies against COX subunit I (A-6403; Molecular Probes; 1:250); COX subunit IV (A-6431; Molecular Probes; 1:1500); porin (A-21317; Molecular Probes; 1:250) alkaline phosphatase-conjugated rabbit anti-mouse immunoglobins (Dako, Golstrup, Denmark; 1:5000). Images were analysed by densitometry with image analysis software (Molecular Analyser, Bio-Rad, Hercules, CA). Coomassie staining of the acrylamide gel was performed, to show equal protein loading.

Determination of proliferation index
Immunostaining on paraffin-embedded sections (2 μm) for Ki-67 was carried out with the antibody clone MIB-1 (Dako Cytomation, Carpinteria, CA) according to the manufacturer’s instruction. The Ki-67 labelling indices were determined by light microscopy with a 40× objective. All nuclei with a visible staining reaction were registered as positive regardless of staining intensity or quality (granular or diffuse). In each section at least 800 nuclei were counted and the Ki-67 labelling indices was defined as the percentage of positively stained nuclei.

Statistical analysis
Significance of the differences among renal carcinoma and control tissues was examined by the Wilcoxon t-test. Comparison of relative enzyme activity and mitochondrial DNA levels to biological features was analysed with the Mann–Whitney or the Kruskal–Wallis test using Prism™ 3.03 software (GraphPad Software Inc., San Diego, CA). A P-value of <0.05 was considered statistically significant.

Results
Mitochondrial DNA content
The median relative ratio of mitochondrial DNA to 18S rDNA (m/r ratio) of the controls (n = 37) was 2.3. The average m/r ratio of all renal carcinoma tissues (n = 37) of 0.7 lies significantly under the average m/r ratio of controls (P < 0.0001; Figure 1A and Table I).

Only one conventional grade 3 renal carcinoma showed a 1.7-fold higher level of mitochondrial DNA compared with the matched controls. The collecting duct carcinoma showed a 62% reduction of mitochondrial DNA, which was in range with the other renal carcinomas investigated (Table I).

The inter-assay variability of the quantitative Southern blot analysis ranged from 5 to 20%.

![Fig. 1](image-url)
Analysis of the content of mitochondria in renal carcinoma

In order to determine if the observed low mitochondrial DNA content in renal carcinoma is due to a reduction of the amount of mitochondria per cell, 15 renal carcinoma tissue sections were analysed by immunofluorescence with an antibody directed against mitochondria. The sections showed intense mitochondrial staining in the control and variable intensity in renal carcinoma tissues (Figure 2). In contrast, immunofluorescence using an antibody directed against COX subunit I revealed an intense signal only in control kidney tissues (Figure 2).

Western blot analysis

The effect of the mitochondrial DNA reduction on the expression of mitochondrial and nuclear-encoded COX subunits was investigated by western blot analysis. In renal carcinoma tissues \( n = 10 \) a significant reduction of nuclear and mitochondrial-encoded subunits was found compared with controls (Figure 3), which is in agreement with the measurement of the COX enzyme activity (Table I). In addition, the median reduction of mitochondrial-encoded subunit I of COX (69%, range 40–93)

**Table I.** Median mtDNA content and enzyme activities in % of control kidney

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>( n )</th>
<th>mtDNA</th>
<th>Median</th>
<th>Range</th>
<th>CS</th>
<th>Median</th>
<th>Range</th>
<th>ICDH</th>
<th>Median</th>
<th>Range</th>
<th>COX</th>
<th>Median</th>
<th>Range</th>
<th>CII</th>
<th>Median</th>
<th>Range</th>
<th>CV</th>
<th>Median</th>
<th>Range</th>
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<tr>
<td>Conv. RC grade 1 + 2</td>
<td>17</td>
<td>26</td>
<td>6–84</td>
<td>51</td>
<td>14–108</td>
<td>28</td>
<td>9–140</td>
<td>20</td>
<td>8–69</td>
<td>18</td>
<td>6–107</td>
<td>25 ( b )</td>
<td>0–159 ( b )</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Conv. RC grade 3 + 4</td>
<td>12</td>
<td>50</td>
<td>7–170</td>
<td>74</td>
<td>4–577</td>
<td>29</td>
<td>3–245</td>
<td>20</td>
<td>9–79</td>
<td>13</td>
<td>2–137</td>
<td>19 ( c )</td>
<td>0–154 ( c )</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Papillary RC</td>
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<td>9</td>
<td>5–91</td>
<td>63</td>
<td>31–75</td>
<td>15</td>
<td>15–23</td>
<td>39</td>
<td>19–78</td>
<td>21</td>
<td>10–44</td>
<td>36 ( d )</td>
<td>13–53 ( d )</td>
<td></td>
<td></td>
<td></td>
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<td>Sarcomatoid RC</td>
<td>2</td>
<td>46</td>
<td>36–56</td>
<td>71</td>
<td>64–77</td>
<td>69</td>
<td>68–69</td>
<td>49</td>
<td>27–70</td>
<td>28</td>
<td>19–38</td>
<td>0</td>
<td>0–0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Collecting duct RC</td>
<td>1</td>
<td>38</td>
<td>n.a.</td>
<td>8</td>
<td>n.a.</td>
<td>4</td>
<td>n.a.</td>
<td>15</td>
<td>n.a.</td>
<td>3</td>
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<td>0</td>
<td>n.a.</td>
<td></td>
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\( ^a \) Conv., conventional.  
\( ^b n = 16. \)  
\( ^c n = 10. \)  
\( ^d n = 4. \)  
\( ^e \) Unclassified renal carcinoma with sarcomatoid features.

n.a., not applicable; RC, renal carcinoma; mtDNA, mitochondrial DNA; CS, citrate synthase; ICDH, isocitrate dehydrogenase; CII, complex II; CV, complex V.

**Fig. 2.** Immunofluorescence analysis of controls and conventional renal carcinomas performed in patient A (A–D) and patient B (E–H) with an antibody against: (A and E) COX subunit I protein on renal control section; (B and F) COX subunit I protein on renal carcinoma sections; (C and G) mitochondrial porin protein on renal control section; (D and H) mitochondrial porin protein on renal carcinoma section. (B1 and F1) is the according HE (haematoxylin and eosin) staining (\( \times 400 \)).

**Fig. 3.** Western blot analysis of COX subunits I and IV (A) and porin (B) in conventional renal carcinomas and controls. Patient 1: renal carcinoma tissue (lane 1) and renal control tissue (lane 2); patient 2: renal carcinoma tissue (lane 4) and renal control tissue (lane 3); patient 3: renal carcinoma tissue (lane 6) and renal control tissue (lane 5); COX I, 57 kDa; COX IV, 19.6 kDa; porin, 31 kDa. (C) Coomassie blue stain of a gel loaded in parallel with the same sample volumes as the gels for western blotting, n.s., non-specific.

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was in the same range as the reduction of the nuclear-encoded subunit IV of COX (55%, range 24–78). In contrast, porin, which is used as a marker for mitochondrial content (23), was not altered in the tumour tissues compared with matched control kidney (Figure 3B).

**Enzyme activities in renal carcinoma**

Table I lists the results of the relative enzyme activities of an entirely nuclear-encoded OXPHOS enzyme (complex II), two OXPHOS enzymes, which are encoded by both the mitochondrial and the nuclear genome (COX, complex V) and of two enzymes of the Krebs cycle (citrate synthase, isocitrate dehydrogenase). All values are means of at least two independent measurements. The inter-assay variability was <20%. Enzyme activities of control kidney showed large individual variations (Figure 1B–F).

Thirty-four of thirty-seven renal carcinoma tissues displayed a 2–10-fold reduction of the enzyme activities of complex II, COX, complex V and isocitrate dehydrogenase, while citrate synthase was less affected (Figure 1 and Table I).

However, one case of grade 3 conventional renal carcinoma showed close to normal activities of COX and complex II and a 3-fold higher activity of the Krebs cycle enzymes citrate synthase and isocitrate dehydrogenase together with a 1.7-fold increase of mitochondrial DNA. Another grade 3 conventional renal carcinoma tissue also displayed a 2–3-fold induction of citrate synthase and isocitrate dehydrogenase accompanied by mitochondrial DNA reduction and low COX and complex II, which was in the range of all other renal carcinomas investigated. The third case was a grade 1 tumour with normal values compared with renal control tissue, except for a 1.5-fold increase in isocitrate dehydrogenase and complex V enzyme activity.

Since these three cases did not follow the trend of the other 34 carcinoma specimens, we used the median instead of the mean values for all of our calculations.

The collecting duct carcinoma showed a severe reduction in the lower range of all analysed enzyme activities (Table I).

The comparison of aurovertin- and oligomycin-sensitive ATPase activity in 10 low grade conventional renal carcinomas (grade 1 and 2) showed that the relative decrease of the ATPase activity of complex V in the tumour tissues compared with the matched control tissues was in the same range (oligomycin 83 ± 13% decrease; aurovertin B 77 ± 20% decrease). Oligomycin- as well as aurovertin-sensitive ATPase activities were lower in tumour samples compared with normal kidney in all samples investigated.

**Correlation of enzyme activities to tumour type and biological features**

In order to evaluate a possible correlation of the mitochondrial energy metabolism to the proliferative activity of the tumour, the median proliferation index was calculated by Ki-67 staining. The index was 2.0 (range 0–68, n = 37) in renal carcinoma and 0.0 (range 0–7.7, n = 13) in controls. No significant correlation of the proliferation index of the tumours to OXPHOS enzyme activity and mitochondrial DNA content could be detected (Figure 4A and D).

Furthermore, there was no significant difference between carcinomas with renal vein invasion (n = 11), distant metastases (n = 8) and without metastases (n = 18) (Figure 4B and E and Table II). In addition, no differences of OXPHOS activities and mitochondrial DNA levels were seen in tumours smaller than 7 cm in diameter (n = 26) or larger (n = 11) (Figure 4C and F and Table II).

We analysed the ploidy of six conventional renal carcinoma tissues by DNA cytometry. Four out of six tumour tissues had a slightly increased ploidy, compared with control tissue. The presence of aneuploid tumour cells in the specimens did correlate with the Ki-67 proliferation index but not with the extent of changes of the mitochondrial energy metabolism.
In the present study we found a significant reduction of enzymes of mitochondrial energy metabolism in 34 of 37 renal carcinomas compared with matching control kidneys. The tumour specimens also showed a severe reduction of the mitochondrial DNA content. In addition, we were able to analyse one case of a rare and highly malignant collecting duct carcinoma. This type of carcinoma originates from the collecting duct in contrast to the other types of renal carcinomas investigated, which arise from the epithelium of the proximal renal ducts and showed a pronounced reduction of all mitochondrial parameters compared with the other renal carcinomas investigated. The decrease of the enzyme activities was still within the range of the other types of renal carcinomas. As we cannot draw a conclusion from one single case, the analysis of further samples of collecting duct carcinoma should elucidate if this specific renal carcinoma shows a different mitochondrial energy metabolism than the other types of renal carcinomas.

Correlation of low respiratory chain content with tumour aggressiveness in renal carcinoma has been reported recently (11). In this previous study, enzyme activities of the mitochondrial energy metabolism of high (3 + 4) and low (1 + 2) grade conventional renal carcinomas were decreased to a comparable extent as in our study with the exception of complex V activity. Simonnet et al. reported that complex V activities, measured enzymatically as aurovertin-sensitive ATPase, were reduced in high grade but normal in low grade conventional renal carcinomas. This is in contrast with our results where we found comparable median low activity of complex V in low grade (25% of control kidney) and high grade (19% of control kidney) conventional renal carcinomas by measurement of oligomycin-sensitive ATPase activity, which determines the activity of assembled FoF1-ATPase, the form of the enzyme necessary in the energy metabolism. In comparison, measurements of aurovertin-sensitive ATPase in 10 cases of low grade conventional renal carcinomas also revealed a reduction of aurovertin-sensitive ATPase in all cases. In the former study (11), the level of complex V protein determined by 2D gel electrophoresis was found to be reduced in low grade (20% of control kidney) and in high grade (27% of control kidney) renal carcinomas. However, the same study showed in low grade renal carcinomas that the mean activity of aurovertin-sensitive ATPase was even slightly elevated compared with control kidney. This result was due to highly elevated ratios in two of ten patients. A similar discrepancy was found in the previous study for papillary renal carcinomas where unchanged activities of aurovertin-sensitive ATPase were found along with a reduction of complex V protein (34% of control kidney). In our study, where we also included five papillary renal carcinomas, the median activity of complex V was significantly reduced (36% of control kidney).

Our results and the data from 2D analysis of the previous study, question the quantification of complex V enzyme activity reported previously (11). The measurement of complex V activity is problematic because the enzyme activity is measured by the use of helper enzymes (pyruvate kinase and lactate dehydrogenase), which must get in the vicinity of the enzymes evenly in contact to achieve a significant better standardization of the measurement of complex V activity (data not shown). Features of progression of renal carcinoma such as the mitotic index, metastasis, tumour size and ploidy were not found to be related to the extent of alterations of mitochondrial energy metabolism and the reduction of mitochondrial DNA levels. Although sarcomatoid renal carcinomas have a poor prognosis, the two unclassified carcinomas with sarcomatoid alterations displayed even less reduced enzyme activity compared with the other renal carcinoma tissues investigated. As we could not find a clear distinction between carcinoma types and aggressiveness, it seems unlikely that alterations of the mitochondrial energy metabolism are strongly associated with the outcome of patients with renal carcinomas.

Since in our Southern blot analysis relative mitochondrial DNA levels are correlated to nuclear DNA, aneuploidy of tumour cells would falsify the relative mitochondrial DNA content per cell. The tumour tissues had only a slightly higher overall ploidy compared with controls, which does not significantly influence the m/r ratio in the renal carcinoma tissues investigated and therefore no correction of m/r data regarding ploidy has been carried out. Down-regulation of the mitochondrial DNA content and the enzyme activities of the mitochondrial energy metabolism seem to be a co-ordinated process. This is evident by the fact that COX, which is partially encoded by the mitochondrial DNA, is diminished to the same extent as complex II, an enzyme of the respiratory chain, which is entirely encoded by the nuclear genome. In contrast, mutations in the deoxyguanosine kinase gene lead to a mitochondrial DNA depletion (24), which results in enzymatic defects of enzymes that contain mitochondrial-encoded subunits but normal activities of the other enzymes-encoded exclusively by the nuclear genome.
genome. Interestingly, the down-regulation of citrate synthase is less pronounced than the decrease of other enzymes measured. This might be due to additional functions of the Krebs cycle enzymes for example in the amino acid metabolism. This should be considered when using citrate synthase as a marker for mitochondrial energy metabolism.

In agreement with an earlier report (25) where the amount of mitochondria was examined by electron microscopy, no obvious reduction of the mitochondrial marker enzyme porin was found in western blot analysis but a clear decrease of the mitochondrial and nuclear-encoded COX subunits comparing renal carcinoma with control kidney (Figure 3). Other studies, however, found variable or decreased content of mitochondria in renal carcinoma tissue (26–28).

In conclusion, we found no correlation of any of the enzymes of the mitochondrial energy metabolism investigated with carcinoma type and aggressiveness. These findings indicate that mitochondrial alterations occur early in the progression process of cancer. In general, low mitochondrial activity rather seems to be an adaptation to environmental conditions of solid tumours, which have to endure hypoxia during their development. Low mitochondrial activity leads to lower oxidative stress under hypoxic conditions and might therefore represent an advantage for carcinoma progression.

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References


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