Loss of Complex I due to Mitochondrial DNA Mutations in Renal Oncocytoma

Johannes A. Mayr,1 David Meierhofer,1 Franz Zimmermann,1 Rene Feichtinger,1 Christian Kögl,3 Manfred Ratschek,3 Nikolaus Schmeller,2 Wolfgang Sperl,1 and Barbara Kofler1

Abstract Purpose: Many solid tumors exhibit abnormal aerobic metabolism characterized by increased glycolytic capacity and decreased cellular respiration. Recently, mutations in the nuclear encoded mitochondrial enzymes fumarate hydratase and succinate dehydrogenase have been identified in certain tumor types, thus demonstrating a direct link between mitochondrial energy metabolism and tumorigenesis. Although mutations in the mitochondrial genome (mitochondrial DNA, mtDNA) also can affect aerobic metabolism and mtDNA alterations are frequently observed in tumor cells, evidence linking respiratory chain deficiency in a specific tumor type to a specific mtDNA mutation has been lacking.

Experimental Design: To identify mitochondrial alterations in oncocytomas, we investigated the activities of respiratory chain enzymes and sequenced mtDNA in 15 renal oncocytoma tissues.

Results: Here, we show that loss of respiratory chain complex I (NADH/ubiquinone oxireductase) is associated with renal oncocytoma. Enzymatic activity of complex I was undetectable or greatly reduced in the tumor samples (n = 15). Blue Native gel electrophoresis of the multi-subunit enzyme complex revealed a lack of assembled complex I. Mutation analysis of the mtDNA showed frame-shift mutations in the genes of either subunit ND1, ND4, or ND5 of complex I in 9 of the 15 tumors.

Conclusion: Our data indicate that isolated loss of complex I is a specific feature of renal oncocytoma and that this deficiency is frequently caused by somatic mtDNA mutations. A shift in cellular energy production from aerobic oxidation in mitochondria to anaerobic glycolysis is a fundamental property of cancer cells, also called the Warburg effect (1, 2). Otto Warburg postulated that damage of the aerobic energy metabolism is a primary and irreversible event in tumor formation (2). Recently, this hypothesis has been supported by the demonstration that mutations of single enzymes of the mitochondrial energy metabolism are associated with tumorigenesis (3, 4). Germline mutations in distinct subunits of succinate dehydrogenase predispose to hereditary paragangliomas and pheochromocytomas (4, 5). Germline mutations in the fumarate hydratase gene can either cause leiomyoma, leiomyosarcoma, and renal cell carcinoma (3) or Leydig cell tumor (6). Therefore, succinate dehydrogenase and fumarate hydratase are regarded as mitochondrial tumor suppressors for these types of tumors (3).

Mitochondrial DNA (mtDNA), the small genome of the mitochondrion, is essential for aerobic energy metabolism and encodes some of the subunits of respiratory chain complexes I, III, and IV, as well as the F1,F0-ATP synthase. Complex I consists of 46 different subunits, with a molecular mass totaling 980 kDa. Seven of these subunits (ND1–ND6 and ND4L) are encoded by mtDNA; the other complex I genes are located on nuclear chromosomes.

Because of its essential role in energy metabolism, the mitochondrial genome has long been suspected of contributing to metabolic alterations in tumors. Such investigations date back to the 1960s, and in fact, numerous somatic mtDNA mutations have been reported in various types of human tumors (7–10). Evidence that dysfunction of enzymes of the respiratory chain, resulting from mutations in mtDNA, plays a role in tumorigenesis is based on in vitro data of cultured cells but has not been shown directly in tumor tissues (10, 11).

Recently, Gasparre et al. have reported that somatic mutations of mtDNA encoded subunits of complex I are statistically significantly associated with thyroid oncocytoma (12). However, no data of the functional consequence on complex I activity of these potentially disruptive mutations have been available.
presented (12). No other direct association of somatic mtDNA mutations with a distinct tumor type and pathophysiology has been published thus far (5, 13).

Renal oncocytomas are usually benign tumors accounting for ~5% of all renal cell neoplasias. A characteristic feature of oncocytomas is the accumulation of a large number of mitochondria (14). This hyperproliferation of mitochondria was first found in thyroid tumors (Hurthle cell carcinoma) and was called an oxyphilic change in the cells. Oxyphilic tumors are also known for various other organs; however, the cause of the hyperproliferation of mitochondria is unknown. In analogy to other known defects of the mitochondrial energy metabolism, a compensatory feedback mechanism leading to proliferation of this organelle has been proposed (15).

Previous studies using restriction length polymorphism analysis have provided contradictory data on mtDNA alterations in renal oncocytomas (16–18), although to our knowledge a systematic search for mutations in the mitochondrial genome has not been reported. Thus, proof of a direct association of mtDNA mutations, biochemistry, and tumorigenesis has been lacking. To elucidate the cause of the mitochondrial alterations in oncocytomas, we investigated the activities of respiratory chain enzymes and screened for mtDNA mutations in renal oncocytomas.

Materials and Methods

Patients. Fifteen renal oncocytoma tissue samples (mean age 61.5 y, range 29-77 y; 66% male) were obtained from the Biobank of the Medical University of Graz and Department of Urology, University Hospital Salzburg. All tissues were frozen and stored in liquid nitrogen within 20 min after surgery. Tumor cell content and cellular composition of samples were evaluated using H&E-stained frozen sections. Corresponding unaffected kidney tissue was available from patients 6, 8, 12, 13, 14, and 15. In addition, 14 age-matched nonneoblastic kidney samples (mean age 61.8 y, range 35-74 y; 60% male) were included as controls for enzymatic measurements (19).

The study was approved by the ethical committee of Medical University of Graz.

Spectrophotometric detection of enzyme activities. Renal oncocytoma and control kidney tissues (20-100 mg) were homogenized with a tissue disintegrator (ULtraturrax, IKA) in extraction buffer [20 mmol/L Tris-HCl (pH 7.6), 250 mmol/L sucrose, 40 mmol/L KCl, 2 mmol/L EGTA] and finally homogenized with a motor-driven Teflon-glass homogenizer (Potter S, Sartorius). The homogenate was centrifuged at 600 × g for 10 min at 4°C. The postnuclear supernatant containing the mitochondrial fraction was used for measurement of enzyme activities, Blue Native PAGE (BN-PAGE) and immunoblot analysis. The rotenone-sensitive complex I activity was measured spectrophotometrically as NADH/acylubiquinone oxidoreductase at 340 nm (20).

The enzyme activities of citrate synthase, complex IV (ferrocytochrome c/oxygen oxidoreductase), and the oligomycin-sensitive ATPase activity of the F1F0 ATP synthase were determined as reported previously (19).

BN-PAGE and immunoblot analysis. Solubilized mitochondrial membranes were prepared from post nuclear supernatant (30 μg protein) of kidney and oncocytoma tissues, as described previously (21). Briefly, post nuclear supernatants were sedimented by centrifugation at 13,000 × g for 15 min. Membranes were solubilized with 1.5% laurylmaltoside for 15 min and centrifuged for 20 min at 13,000 × g. Solubilized membranes were loaded on a 5% to 13% polyacrylamide gradient gel and separated electrophoretically. The in-gel activity assay of complex I and cytochrome c oxidase were done as previously described (22).

For immunoblot analysis, membrane preparations were separated by BN-PAGE (5-13%) and blotted onto polyvinylidene difluoride membrane (Hybond-P, GE Healthcare) using a CAPS buffer [10 mmol/L 3-[cyclohexylaminol]-1-propane sulfonic acid (pH 11), 10% methanol]. The membrane was washed in 100% methanol for 2 min and blocked for 1 h at room temperature in 5% fat-free milk powder dissolved in TBS. The primary antibody, diluted in 1% milk powder-TBS, was added overnight at 4°C. The following primary antibody dilutions were used: complex I subunit NDUFa9 monoclonal antibody (1:480; MitoSciences) and complex V subunit α monoclonal antibody (1:100,000; MitoSciences). After extensive washing, blots were incubated for 2 h with antiimmunoglobulin (IgG) POD-labeled antibody (Lumi-LightPLUS Western blotting kit, Roche) diluted 1:400. Detection was carried out with Lumi-LightPLUS POD substrate (Roche). After detection of complex I subunit NDUFa9, the polyvinylidene difluoride membrane was washed twice in stripping buffer [25 mmol/L Glycin-HCl (pH 2), 2% SDS] for 10 min, and a subsequent immunodetection with complex V subunit α antibody was done as described above.

mtDNA analysis. DNA was isolated from tissues by proteinase K digestion followed by phenol/chloroform extraction. The mtDNA of ND and tRNA genes was amplified using overlapping PCR fragments (23). All samples were sequenced using a GenomeLab SNP-Primer Extension kit (Beckman Coulter). Mutations were analyzed with the Beckman software investigator. Exact positions of mutations and amino acid changes were defined with the mitoAnalyzer tool (MitoAnalyzer 2000)4, using the mtDNA Genbank sequence J01415.1 as reference.

Determination of mutational load of the A3243G mutation. The percentage of mutational load was quantified by densitometry of ethidium bromide–stained agarose gels, as described previously (24).

Results

Enzymatic and immunoblot analyses of the respiratory chain in renal oncocytomas. The enzyme activities of complexes I, IV, and V of the respiratory chain, which consist of mitochondrial and nuclear encoded subunits, and citrate synthase, an enzyme of the Krebs cycle, were investigated in postnuclear supernatants from 15 oncocytoma tissues. Activities of complexes IV and V, as well as citrate synthase in the oncocytomas, were found to be increased 3-fold to 7-fold, compared with unaffected kidney cortex samples (Fig. 1; Table 1). In striking contrast, the activity of complex I was below the detection limit of <3 nmol/min/mg protein in eight oncocytomas (Fig. 1; Table 1). The other samples showed a residual activity of complex I of <25% compared with kidney control samples (Table 1). If the samples showing residual complex I activity are compared with the activity of citrate synthase, a marker enzyme of mitochondrial energy metabolism (25), the relative enzyme activity of complex I, is <5% compared with normal kidney. This striking reduction of complex I activity was also found by activity-staining of BN-PAGE (Fig. 2A and Supplementary Fig. S1). None of the tumor samples showed any detectable activity compared with their corresponding tumor tissue. Activity-staining of cytochrome c oxidase underlines the up-regulation of complex IV in renal oncocytoma as measured photometrically (Fig. 2B).

Immunoblot analysis of BN-PAGE samples revealed lack of assembled complex I (Fig. 2C) in the tumor tissues, whereas staining of the same blot with complex V antibodies reflects the high enzyme activity of this complex measured photometrically (Fig. 2D).

4 http://www.cstl.nist.gov/biotech/strbase/mitoanalyzer.html
Mutation analysis of the mtDNA in renal oncocytomas. To elucidate the genetic basis for the loss of complex I, we sequenced all seven mitochondrially encoded complex I genes and noticed frame-shift mutations in either the ND1, ND4 or ND5 gene in 9 of the 15 tumors with no detectable wild-type mtDNA (Table 1; Fig. 3). Insertion of a cytosine residue into a stretch of six cytosines at mtDNA positions 3566 to 3571 was detected in the ND1 gene in tumor tissues of cases 1, 2, and 14 (Table 1). In case 3, a deletion of one cytosine in the same polycytosine stretch was identified. All four of these mutations cause termination of translation in the first third of the protein. In case 4, we noticed an insertion of a cytosine residue in a stretch of six cytosines at positions 10947 to 10952 of the ND4 gene. This insertion causes a frame-shift and creates a stop codon ~150 bp downstream, which results in a truncated ND4 protein. In cases 5 and 6, deletions of one adenine in a stretch of seven adenine residues (11032-11038) were found, which results in premature termination of translation of ND4. Two other insertion mutations—in cases 7 and 8— affect the same stretch of six cytosine residues (12385-12390) of the ND5 gene. In case 7, an insertion of one thymine directly before the homopolymeric sequence was identified; in case 8, an extra cytosine was added to the polycytosine run. Both of these frame-shift mutations create a stop codon within the first 10% of the ND5 gene. Remarkably, tissue 7 and tissue 1 originate from the same patient with two independent oncocytomas. Oncocytoma number 7 was excised from the right kidney 18 months after oncocytoma number 1 was surgically removed from the left kidney. To exclude the possibility of sample mix-up, we also sequenced the displacement loop of the mtDNA of these samples and found identical sequences in both, confirming that they are indeed derived from the same individual. The matching normal kidney cortex of cases 6, 8, and 14 revealed no preexisting mutation (Fig. 3). A leucine-to-proline substitution caused by a transition mutation at nucleotide position 13493 was observed in case 9 (Table 1). The effect of this amino acid change on the structure of the ND5 subunit is not known, although the alteration could be severe enough to disrupt the catalytic activity of the entire complex. Finally, an A-to-G transition at position 3243 in the gene encoding tRNALeu(UUR) was detected in case 13. RFLP analysis revealed >95% mutational load in the oncocytoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Complex I</th>
<th>Citrate Synthase</th>
<th>Complex IV</th>
<th>Complex V</th>
<th>mtDNA mutation*</th>
<th>Affected genes</th>
<th>Protein change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;3</td>
<td>791</td>
<td>205</td>
<td>481</td>
<td>3571_3572insC</td>
<td>ND1</td>
<td>Leu89fs</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>1.656</td>
<td>1.393</td>
<td>107</td>
<td>3571_3572insC</td>
<td>ND1</td>
<td>Leu89fs</td>
</tr>
<tr>
<td>3</td>
<td>&lt;3</td>
<td>727</td>
<td>285</td>
<td>n.d.</td>
<td>3571delC</td>
<td>ND1</td>
<td>Leu89fs</td>
</tr>
<tr>
<td>4</td>
<td>&lt;3</td>
<td>316</td>
<td>336</td>
<td>1375</td>
<td>10952_10953insC</td>
<td>ND4</td>
<td>Leu65fs</td>
</tr>
<tr>
<td>5</td>
<td>&lt;3</td>
<td>1.565</td>
<td>323</td>
<td>71</td>
<td>11038delA</td>
<td>ND4</td>
<td>Lys93fs</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>684</td>
<td>632</td>
<td>359</td>
<td>11038delA</td>
<td>ND4</td>
<td>Lys93fs</td>
</tr>
<tr>
<td>7</td>
<td>&lt;3</td>
<td>324</td>
<td>151</td>
<td>268</td>
<td>12384_12385insT</td>
<td>ND5</td>
<td>Pro17fs</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>1.184</td>
<td>298</td>
<td>n.d.</td>
<td>12390_12391insC</td>
<td>ND5</td>
<td>Ile19fs</td>
</tr>
<tr>
<td>9</td>
<td>&lt;3</td>
<td>481</td>
<td>142</td>
<td>276</td>
<td>T13493C</td>
<td>ND5</td>
<td>Leu386Pro</td>
</tr>
<tr>
<td>10</td>
<td>&lt;3</td>
<td>975</td>
<td>292</td>
<td>n.d.</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>11</td>
<td>&lt;3</td>
<td>456</td>
<td>188</td>
<td>n.d.</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>275</td>
<td>232</td>
<td>306</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>13</td>
<td>10</td>
<td>910</td>
<td>604</td>
<td>246</td>
<td>A3243G</td>
<td>tRNALeu(UUR)</td>
<td>—</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>638</td>
<td>461</td>
<td>399</td>
<td>3571_3572insC</td>
<td>ND1</td>
<td>Leu89fs</td>
</tr>
<tr>
<td>15</td>
<td>11</td>
<td>753</td>
<td>952</td>
<td>303</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Oncocytoma, mean ± SD 3 ± 1 782 ± 109 433 ± 89 381 ± 106 Kidney, mean ± SD (n = 20) 46 ± 5 111 ± 9 157 ± 17 56 ± 6

NOTE: Enzyme activity (nmol/min/mg protein) of complex I, citrate synthase, complex IV, and complex V. Abbreviation: n.d., not determined (lack of sample).

*Positions of mutations refer to the mtDNA sequence Genbank accession number J01415.1.
tissue, but the mutation was also detectable in the normal kidney tissue with a 20% mutational load (data not shown). Interestingly, the same mutation is a frequent cause of MELAS (mitochondrial myopathy, encephalomyopathy, lactic acidosis, and stroke-like episodes) syndrome. In the remaining four cases, screening of all mtDNA-encoded complex I and mitochondrial tRNA genes did not reveal any somatic mutation with pathogenic potential.

Discussion

Here, we provide the first report that renal oncocytomas show a loss of complex I of the respiratory chain, which is frequently caused by mutations of the mtDNA. Somatic mutations of the mtDNA have been reported in a number of different tumor types (7, 8, 13). However, most of these mutations were silent without an apparent pathogenic potential and a role for mtDNA in tumor development has not been established yet (5). Recently, an association of somatic mtDNA mutations with the oncocytic phenotype of thyroid tumors has been reported (12). Two types of frame-shift mutations (3571_3572insC; 11038delA) detected in thyroid oncocytomas have also been detected in the present study in renal oncocytoma tissues. Our data now provide also the functional consequence of these mutations by showing loss of enzyme activity and assembly of complex I.

We note that a previous study found an ~ 50% reduction in complex I activity in renal oncocytomas (26). Residual complex I activity found in that study may have been due to contamination of investigated tumor tissues with normal kidney tissue and epithelial cells of vessels. To avoid such contamination, we validated cryostat sections of tumor tissues histologically before using adjacent sections for biochemical and genetic investigations. In addition to photometric measurement of complex I, we substantiated our findings by complex I activity-staining and immunoblot analysis of Blue Native gels.

The biochemical consequences of the A3243G mutation of case 13 are in agreement with the decreased activity of complex I reported in muscle biopsies and fibroblasts of patients carrying this mutation (27). The A3243G mutation might be sporadically associated with other tumor types because it was also detected in a colon cancer sample (28) and a renal cell carcinoma (24).

The frame-shift mutations detected in this study are predicted to cause termination of translation in the first third of the corresponding proteins. Similar mutations are known to affect not only the enzyme activity, but also the assembly of complex I (29), which is consistent with our biochemical and immunoblot data. Homopolymeric stretches (5-8-bp long) in mtDNA are known hotspots for base pair insertions and deletions (30). Interestingly, several of these mtDNA repeat mutations were reported in different tumors in earlier studies (Fig. 4). Because no respiratory chain activity measurements were reported in those tumors, it is difficult to evaluate the effect of those mutations on mitochondrial energy metabolism.
and their role in tumorigenesis, especially in cases of high mutation load.

We hypothesize that the four oncocytomas in our study with no detected pathogenic mutations in mtDNA (cases 10–12 and 15; Table 1) may have mutations in 1 of the 39 known nuclear genes encoding complex I subunits or assembly factors (31). Loss of heterozygosity in combination with such a nuclear mutation would be expected to lead to impairment of complex I, and observations of frequent cytogenetic abnormalities in oncocytomas lend support to this idea (32).

Oncocytomas occasionally appear in both kidneys. Interestingly, in one such case, distinct cytogenetic backgrounds were found in each tumor (32). A similar case with two independent oncocytomas was included in our study, wherein oncocytomas were excised first from the left (case 1) and then from the right kidney (case 7) over an interval of 18 months. As in the aforementioned study (32), these tumors were distinct from each other, and they carried different frame-shift mutations in the mtDNA. We can only speculate as to whether the induction of these tumors is due to an environmental trigger or to a genetic predisposition in such patients.

Patients with Birt-Hogg-Dube syndrome have an increased incidence of renal tumors, including renal oncocytomas (33). However, a function for the BDH (folliculin) gene, which is thought to be responsible for this syndrome, has not yet been determined. One possibility is that this protein is involved in cellular energy metabolism (34). Recently, somatic missense mutations in the complex I assembly protein GRIM-19 were identified in 15% of sporadic oxyphil Hurthle cell tumors of the thyroid (35), suggesting that impairment of complex I activity may be associated with oxyphilic tumors generally and not just renal oncocytomas. Further biochemical and genetic support for this possibility comes from the recent description of a combined reduction in the activities of complexes I and III in a cell line derived from an oxyphilic thyroid follicular carcinoma. The reduction of complex I activity in that tumor was caused by a frame-shift mutation in the ND1 gene (36), which is also what we observed in cases 1 and 2 of our study.

Recently, it was shown that complex I is an integral component of apoptotic pathways. The nuclear encoded subunit NDUFS1, which is part of the iron-sulfur clusters of complex I, is proteolytically cleaved by caspase-3 (37). Therefore, complex I seems to have a dual function—creating a proton gradient under normal conditions and participating in apoptosis after caspase-3 cleavage. Loss of this intermediate step of apoptosis due to a deficiency of complex I might lead to a selective advantage for tumor cells. We envisage that in cells lacking functional complex I, apoptosis is attenuated, which in turn would favor tumor formation. In support of this hypothesis, Shidara et al. showed that pathogenic mutations in the mitochondrial ATP76 gene contribute to promotion of cancer by prevention of apoptosis (38).

Our findings reveal that isolated deficiency of complex I besides high activity of the other OXPHOS enzymes can be regarded as a specific feature of renal oncocytoma. Our results also support the hypothesis of Otto Warburg, proclaimed >80 years ago, that irreversible damage to aerobic cellular respiration is an important event in tumor development (2).

Acknowledgments
We thank K. Zatloukal for the critical reading of the manuscript and I. Kufferath and D. Radmayr for technical assistance.

References
9. Czarnecka AM, Golik P, Bartnik E. Mitochondrial...


**Loss of Complex I due to Mitochondrial DNA Mutations in Renal Oncocytoma**


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: <a href="http://clincancerres.aacrjournals.org/content/14/8/2270">http://clincancerres.aacrjournals.org/content/14/8/2270</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://clincancerres.aacrjournals.org/content/suppl/2008/04/21/14.8.2270.DC1">http://clincancerres.aacrjournals.org/content/suppl/2008/04/21/14.8.2270.DC1</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 48 articles, 9 of which you can access for free at: <a href="http://clincancerres.aacrjournals.org/content/14/8/2270.full#ref-list-1">http://clincancerres.aacrjournals.org/content/14/8/2270.full#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citing articles</td>
<td>This article has been cited by 6 HighWire-hosted articles. Access the articles at: <a href="http://clincancerres.aacrjournals.org/content/14/8/2270.full#related-urls">http://clincancerres.aacrjournals.org/content/14/8/2270.full#related-urls</a></td>
</tr>
</tbody>
</table>

**E-mail alerts**  
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.