Respiratory chain complex I is a mitochondrial tumor suppressor of oncocytic tumors

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1. ABSTRACT

Oncocytic tumors, also called oxyphilic tumors, are characterized by hyperproliferation of mitochondria, which histologically presents as a fine granular eosinophilic cytoplasm. In accordance with the high mitochondrial density in oncocytomas, transcript levels of subunits of complexes of the oxidative phosphorylation (OXPHOS) system are increased. Hence, for a long time oncocytomas were presumed to have a highly active aerobic mitochondrial energy metabolism. Recently, detailed analysis of all OXPHOS complexes in a variety of oncocytomas revealed loss of complex I and compensatory up-regulation of the other complexes. In half of the oncocytoma cases examined the absence of complex I is caused by disruptive mutations in mitochondrial DNA encoding complex I subunits. The new data presented here on rare oncocytomas and the accompanying review of the literature clearly indicate that complex I deficiency in combination with up-regulation of mitochondria can be regarded as a hallmark of oncocytic tumor cells. Therefore, complex I of the respiratory chain has to be added to the growing list of mitochondrial tumor suppressors.

2. INTRODUCTION

More than half a century ago, Nobel Prize laureate Otto Warburg posed the hypothesis of aerobic glycolysis, stating that normal cells tend to produce energy by glycolysis only under anaerobic conditions, whereas cancer cells do so even in the presence of oxygen (1). The first hint that a genetic defect of aerobic mitochondrial energy metabolism contributes to tumor formation was found in hereditary paraganglioma and familial pheochromocytoma, by demonstrating that mutations in the *SDHB* or *SDHD* genes, two nuclear encoded subunits of mitochondrial complex II, are associated with the development of these tumors (2) (3). Subsequently, germline mutations in the *fumarate hydratase* (*FH*) gene and reduced fumarate hydratase activity were shown to predispose to hereditary leiomyomatosis, renal cancer, and Leydig cell tumors (4) (5). Recently, glioblastoma multiforme, a very aggressive subtype of glioma, revealed specific point mutations in the *isocitrate dehydrogenase 1 and 2* (*IDH1* and *IDH2*) genes in 12–20% of the cases (6) (7). The same mutations were also found in >70% of astrocytomas, oligodendrogliomas and glioblastomas (8).

These data indicate that the *IDH1, IDH2*, *SDH* and *FH* genes can be regarded as mitochondrial tumor suppressor genes (9) (10).

Besides the nuclear genes encoding SDH, IDH and FH, the mitochondrial genome (mtDNA) also has become of special interest in cancer research (11) (12) (13). The mtDNA, a circular DNA molecule within the mitochondrial matrix, encodes a total of 13 subunits of the respiratory chain, including seven subunits of complex I, one of complex III, three of complex IV and two of the F_1F_0-ATP synthase and 22 transfer RNAs. Over one hundred publications have reported the presence of somatic mtDNA mutations in a wide variety of tumor types (14) (11).

Although somatic mutations are frequent in cancers, their functional significance is uncertain for most tumor types (15). Coller *et al.* have shown by a mathematical approach that these mutations could also arise by chance without conferring any physiological advantage or tumorigenic effect (16) (17). This hypothesis does not apply equally to silent and non-silent mutations. In fact, most variants found in tumors are also present as polymorphic variants in the control population, whereas for those inducing amino acid changes, it is difficult to prove a role in tumor pathology. Therefore, functional investigations of specific alterations of aerobic energy metabolism might be used to identify isolated OXPHOS defects or at least should complement genetic analysis of mtDNA (15) (18).

Oncocyte is the general term applied to cells exhibiting a characteristic phenotype of a fine granular eosinophilic cytoplasm histologically and an increase in the number of mitochondria ultrastructurally (19). The abnormal increase in the amount of mitochondria was confirmed by electron microscopy in different studies (20) (21). The percentage of cytoplasm filled with mitochondria varies between 30% and 60%. This unique feature also led to the cytological designation of 'oxyphilic' tumor cells. In 1991, Ebner *et al.* were among the first to analyze the changes of the mitochondrial energy metabolism in more detail and found by enzyme histochemistry an up-regulation of all OXPHOS complexes in thyroid oncocytomas (22). In 1996, Heddi *et al.* analyzed the expression of nuclear and mitochondrial encoded genes of the respiratory chain in salivary gland oncocytoma and found a specific induction of OXPHOS gene expression as well as increased mtDNA content in oncocytomas, which was in accordance with the ultrastructural findings and the data of Ebner *et al.* (23). Based on transcriptional profiling data of 29 thyroid oncocytomas, Baris *et al.* hypothesized that, unlike most solid tumors, thyroid oncocytomas produce energy through an aerobic pathway (24). The reason for the extraordinary mitochondrial biogenesis in oncocytic cells remained unsolved until Savagner *et al.* reported in 2002 defective mitochondrial ATP synthesis in thyroid oncocytomas (25), suggesting a compensatory mechanism may be responsible for mitochondrial proliferation in response to a defective aerobic energy metabolism. The specific defect and genetic basis still remained unidentified but it became clear that the aberrant biogenesis of mitochondria in oncocytic cells bears intriguing similarities to mitochondrial encephalomyopathies. In the latter conditions, proliferation of abnormal mitochondria in muscle gives a phenotype termed ragged red fibers (RRF), which is a characteristic histochemical finding in skeletal muscle of patients with pathogenic mtDNA mutations (26).

In the present article we focus on recently published and new data regarding the specific alterations of aerobic energy metabolism at the biochemical and genetic levels in oncocytomas of different origintissues.

3. MATERIALS AND METHODS

3.1. Tumor samples

The study population comprised salivary gland oncocytomas (n=13), parathyroid oncocytomas (n=6), pituitary gland oncocytomas (n=2), oncocytoma of the eyelid (n=1), and adrenal gland oncocytoma (n=1). Samples were obtained from the Biobank of the Medical University of Graz and the Department of Pathology, University Hospital of Salzburg. The study was approved by the ethics committee of the Medical University of Graz and was performed according to the Austrian Gene Technology Act and in accordance with the Helsinki Declaration of 1975 (revised 1983) and the guidelines of the Salzburg State Ethics Research Committee, being neither a clinical drug trial nor epidemiological investigation. All patients signed an informed consent concerning the surgical removal and therapy of the tumors. Furthermore, the study did not extend to examination of individual case records. The anonymity of the patients' samples has been ensured.

3.2. Immunohistochemical staining and analysis

The immunohistochemical staining of complex I (subunit NDUFS4, Abcam), complex II (subunit 70 kDa, Mitosciences), complex III (subunit core 2, Mitosciences), complex IV (subunit I, Mitosciences), complex V (subunit alpha, Mitosciences) and porin (31HL, Mitosciences) was performed as described previously (27), with the following change: for heat-induced epitope retrieval, EDTA-T buffer (1 mM EDTA, pH 8.0, 0.05% Tween 20) was used.

3.3. Analysis of mtDNA

Sequence analysis of the mitochondrially encoded subunits of complex I (ND1 to ND6 and ND4L), complex IV (COI to COIII) and mitochondrial tRNAs was performed as described previously (27).

4. RESULTS AND REVIEW OF THE LITERATURE

4.1. Renal oncocytoma

Renal oncocytomas arise from the collecting duct and account for approximately 5% of all renal tumors (28). Renal oncocytomas are homogenous tumors with round cells which have lost their polarity (29). They are considered to be benign, in spite of rare cases of invasiveness.

The first data that complex I activity is decreased in oncocytomas were reported in 2003 by Simonnet *et al.*

Loss of complex I in oncocytic tumors

Table 1. Mutations of mtDNA detected in oncocytomas of different tissues

organ	mtDNA mutation*	protein change	affected gene	Study
Kidney	564G>A	-	D-Loop	(31)
Kidney	3571_3572insC	Leu89fs	ND1	(32)
Kidney	3571delC	Leu89fs	ND1	(32)
Kidney	3664G>A	Gly120X	ND1	(31)
Kidney	4063G>A	Gly244X	ND1	(31)
Kidney	3571_3572insC	Leu89fs	ND1	(31)
Kidney	10952_10953insC	Leu65fs	ND4	(32)
Kidney	11038delA	Lys93fs	ND4	(32)
Kidney	11872_11873insC	Pro371fs	ND4	(31)
Kidney	12384_12385insT	Pro17fs	ND5	(32)
Kidney	12390_12391insC	Ile19fs	ND5	(32)
Kidney	13937delAC	His540fs	ND5	(31)
Kidney	14429delG	Trp87fs	ND6	(31)
Kidney	14754delC	Pro3fs	Cytb	This study
Kidney	15342insT	Phe210fs	Cytb	(31)
Thyroid	3571_3572insC	Leu89fs	ND1	(45)
Thyroid	3331del242bp	Leu9fs	ND1	(45)
Thyroid	3571_3572insC	Leu89fs	ND1	(27)
Thyroid	5185G>A	Trp239X	ND2	(45)
Thyroid	4720G>A	Trp84X	ND2	(45)
Thyroid	11084delCA	Thr109fs	ND4	(45)
Thyroid	11038delA	Lys93fs	ND4	(45)
Thyroid	10885delT	Phe42fs	ND4	(45)
Thyroid	11403G>A	Trp215X	ND4	(45)
Thyroid	10952_10953insC	Leu65fs	ND4	(27)
Thyroid	10477delT	Leu3fs	ND4L	(27)
Thyroid	13414G>A	G360X	ND5	(45)
Thyroid	13870A>T	K512X	ND5	(45)
Thyroid	13235_13236insT	Lys300fs	ND5	(45)
Thyroid	12539G>A	Trp68X	ND5	(27)
Thyroid	13235_13236insT	Lys300fs	ND5	(27)
Thyroid	14339_14340insA	Val112fs	ND6	(27)
Thyroid	14603_14604insT	Ser24fs	ND6	(27)
Parathyroid	3571_3572insC	Leu89fs	ND1	This study
Parathyroid	13031G>A	Trp232X	ND5	This study
Parathyroid	13767delC	Pro477fs	ND5	This study
Parathyroid	14079delA	Gln580fs	ND5	This study
Salivary gland	5107delG	Thr213fs	ND2	This study
Salivary gland	11403G>A	Trp215X	ND4	This study
Salivary gland	11559G>A	Trp267X	ND4	This study
Adrenal gland	11038delA	Lys93fs	ND4	This study
Breast	3331del242bp	Leu9fs	ND1	(45)
Nasopharynx	12425delA	Asn30fs	ND5	(51)
Pituitary gland	11872_11873insC	Pro371fs	ND4	This study

* Positions of mutations refer to the mtDNA sequence Genbank accession number: J01415.2

(30). A reduction of complex I activity to less than 65% compared to adjacent normal tissue has been demonstrated. Interestingly, complex I activity was also reduced in the corresponding normal tissue. Two-dimensional gel electrophoresis of mitochondrial proteins revealed decreased protein content of the nuclear encoded 75 kDa subunit of complex I in renal oncocytomas compared to the corresponding normal tissue (30). Two subsequent independent studies confirmed the complex I deficiency (31) (32)**.** Complex I activity was either below the detection limit or <25% of the level in control kidney tissue (31) (32). Blue native gel electrophoresis of multisubunit enzyme complexes demonstrated a loss of assembled complex I, whereas complex V showed compensatory up-regulation (32). In accordance, deficiency of the complex I subunit NDUFS3 and upregulation of the complex IV subunits COX5A, COX5B and ATP5H from complex V was found by two dimensional gel electrophoresis in renal oncocytoma (33).

Two studies independently reported for the first time a genetic explanation for the striking loss of complex I in oncocytomas (31) (32) (Table 1): they found disruptive mutations (frame shift or nonsense) in subunits of complex I encoded by mtDNA (Table 1).

Gasparre *et al.* found in four of seven cases mutations, which generated a stop codon leading to truncation of the protein near the N-terminus. Additionally, one disruptive mutation was found in the *cytochrome b* gene, resulting in a loss of complex I and reduced complex II/III activity. We also detected in one sample a frame-shift mutation 14754_delC at the beginning of the *cytochrome b* gene, causing a loss of assembled complex III and in addition a deficiency of complex I (Table 1). It is rather astonishing that the absence of complex III causes a deficiency of complex I. Similar observations have been reported by Acin-Perez *et al.* in two cases of cytochrome b

mutations (34). The authors argued that respiratory chain enzymes form supercomplexes and they found that complex I is formed de novo but is unstable in the absence of complex III. Finally, one case was reported to harbor a somatic mutation in the major H strand promoter, resulting in reduced activity of all OXPHOS complexes containing mitochondrial encoded subunits (31).

Mutation analysis of mtDNA by Mayr *et al.* revealed disruptive mutations in mitochondrially encoded complex I subunits in 11 of 15 tumor samples. Frame-shift mutations were found in nine cases, located in the first third of the gene and causing a truncated complex I subunit. A recurring frame-shift mutation in the *ND1* gene at position 3571_3572insC seems to identify this site as a mutational hot spot, accounting for 18% of all mtDNA mutations detected in oncocytomas (Table 1). A leucine-to-proline substitution was detected in one case, possibly severe enough to cause a complex I deficiency. The A3243G transition, known as a frequent cause of the MELAS syndrome, was found in one renal oncocytoma. Interestingly, one patient developed two independent oncocytomas in the left and right kidneys. The oncocytoma from the right kidney harbored a frame-shift mutation in the *ND5* gene, whereas the left kidney had a frame-shift mutation in the *ND1* gene (32).

In oncocytomas where no potential pathogenic mtDNA mutations have been identified, it is most likely that nuclear components of complex I are affected. Nuclear gene sequences encoding and regulating the expression of complex I subunits and their assembly factors have, to our knowledge, not been analyzed in detail in renal oncocytomas. One reason might be the extensive number of candidate genes (>40), which would have to be sequenced.

The specific complex I deficiency in renal oncocytomas is in contrast to the alterations found in other renal tumors, where the Warburg effect is achieved by a significant reduction of all enzymes of the respiratory chain and accompanied by a massive reduction of mtDNA content. This mitochondrial phenotype in renal cell carcinoma can be explained by the loss of the von Hippel Lindau (VHL) gene, which has been shown to induce hypoxia-inducible factor 1 (HIF-1), a major factor contributing to reduced respiration in VHL-deficient cancer cells (35) (36). A general reduction of the OXPHOS complexes and reduced mtDNA copy number was also observed in neuroblastomas, but without alterations of the VHL protein (37).

4.2. Thyroid oncocytoma

Oncocytic thyroid tumors, also known as Huerthle cell tumors, are composed of at least 75% oncocytic tumor cells and account for 3% to 10% of thyroid tumors (38) (39). Oncocytic carcinoma of the thyroid appears to have a worse clinical outcome than non-oncocytic thyroid carcinoma, due to poor responsiveness of oncocytomas to radioiodine therapy (40).

In agreement with the mitochondrial hyperproliferation phenotype, coordinated overexpression of genes involved in mitochondrial biogenesis, such as nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (TFAM), was observed in thyroid oncocytomas (41) (24). Also, death-associated protein 3 (DAP3), one of the constituents of the small subunit of the mitochondrial ribosome, was found to be up-regulated in thyroid oncocytic tumors (42). A first hint that thyroid oncocytomas exhibit a defect in aerobic energy metabolism came from Savagner *et al.*, who showed a 50% lower level of ATP synthesis in thyroid oncocytomas in comparison with the basal rate of normal thyroid tissue (25). Interestingly, measurement of oxygen consumption by polarography of the same samples produced no evidence of OXPHOS defects.

The first nuclear gene mutation specific to oncocytic thyroid tumors was a heterozygous mutation of the nuclear encoded *GRIM-19* gene, which encodes a complex I subunit necessary for assembly of the complex (43). Four of 26 cases of sporadic thyroid oncocytomas revealed heterozygous point mutations in the *GRIM-19* gene. In one patient the mutation was also detected in non-cancerous thyroid tissue, peripheral blood, and in a blood sample from the patient's son, demonstrating the germline nature of the mutation. In none of six cases with known familial thyroid oncocytoma, nor in any of 20 non-oncocytic thyroid carcinomas, were mutations in the *GRIM-19* gene detected (43).

The so-called common deletion (CD) of mtDNA, a 4977-bp deletion affecting the OXPHOS genes *ATPase6*, *ATPase8*, *COIII*, *ND3*, *ND4L*, *ND4* and *ND5*, was detected with a higher prevalence in oncocytic thyroid tumors than in non-oncocytic thyroid tumors (44). Nevertheless, the percentage of mtDNA carrying the common deletion varied between 8% in oncocytic follicular carcinomas to 4% in oncocytic follicular adenomas and papillary carcinomas. The low level of mutational load could not explain the dramatic mitochondrial phenotype of oxyphilic cells.

Gasparre *et al.* found that 12 of 45 (27%) specimens showed disruptive mutations, either frame shift or nonsense, resulting in impaired protein synthesis (Table 1). In contrast to the mutations found in renal oncocytomas, not all mutations were homoplasmic; in some cases the mutation rate varied between 25% to 98% (45). XTC.UC1, a cell line derived from a metastasis of a Huerthle cell carcinoma, has a 3571_3572insC mutation in the *ND1* gene, which was also found in three cases of thyroid oncocytomas. Interestingly, the XTC.UC1 cell line also carries a heteroplasmic (70%) point mutation (15557G>A) in the *cytochrome b* gene, which leads to an amino acid substitution at a non-conserved position (46). In a further 19 thyroid oncocytoma tissues, we used immunohistochemical analysis to verify that these mutations indeed can affect the level of complex I in the thyroid oncocytomas. We found a specific lack of complex I in all 19 cases, whereas other enzymes of mitochondrial energy metabolism showed compensatory up-regulation (27). Subsequent analysis of the mtDNA revealed disruptive mutations (six frame-shift and one nonsense) in complex I subunits in 7 of the 19 (37%) tumors, resulting in
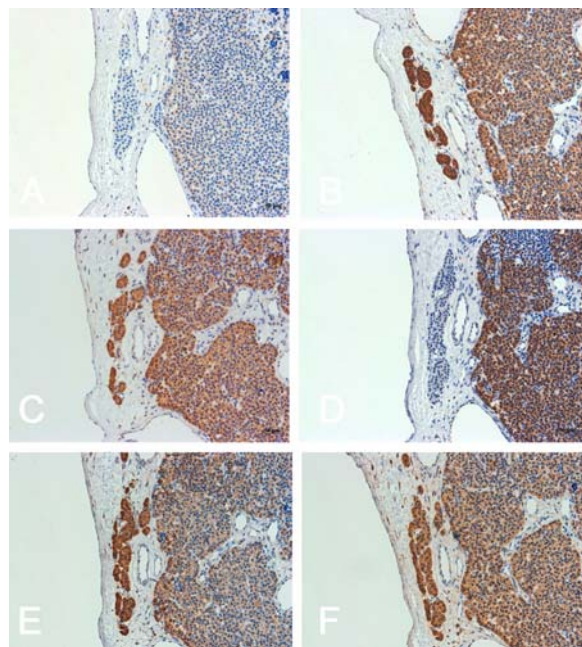
Figure 1. Immunohistochemical staining of all respiratory chain enzyme complexes and porin of a parathyroid adenoma. Expression of respiratory chain complexes I (A), complex II (B), complex III (C), complex IV (D), complex V (E) and porin (F) in a parathyroid adenoma exhibiting complex I deficiency and heterogenous expression of complex IV.

truncated subunits (27) (Table 1). Truncation of the ND subunits prohibits assembly of complex I and seems to lead to degradation of unassembled subunits even though the expression of complex I subunits is up-regulated to a similar extent as the expression of the other OXPHOS complexes in oncocytomas (25).

In contrast to our recent findings (27), Ebner *et al.* previously reported an increase in the enzymatic activity of complex I in nine thyroid oncocytomas (22). However, in this study the activity of complex I was determined by enzyme histochemistry of total NADH dehydrogenases, a method which also detects cytosolic NADH dehydrogenases and therefore can not be regarded as a measure of complex I activity.

4.3. Parathyroid oncocytoma

Oncocytic parathyroid adenomas account for 4% to 8% of all parathyroid adenomas (12). Analysis of the mtDNA in oncocytic parathyroid adenomas revealed nonsense mutations in mitochondrially encoded complex I subunits in 33% (4/12) of the samples studied (47).

Here we present data for six cases of oncocytic parathyroid adenomas (mean age: 63 years; range: 44 - 87; 33% male) for alterations of respiratory chain complexes by immunohistochemical staining and sequence analysis of the mtDNA. Oncocytic parathyroid tumor cells were negative for the complex I subunit NDUFS4 when compared to the adjacent non-cancerous tissue in all

investigated specimens. Immunohistochemical staining of respiratory chain complex II subunit 70 kDa, complex III subunit core 2, complex IV subunit I, complex V subunit alpha, and the mitochondrial membrane protein porin, demonstrated an up-regulation of all of these proteins in all tumor samples compared to the adjacent normal tissue. Sequence analysis of the mitochondrially encoded subunits of complex I revealed disruptive mutations, either frame-shift or nonsense mutations, in the *ND1* or *ND5* gene. Three cases had frame-shift mutations (3571_3572insC, 13767delC, 14079delA) and one case exhibited a nonsense mutation (13031G>A) (Table 1).

In one of the cases, in addition to loss of complex I, a nodular loss of complex IV was found. Because <5% of the tumor cells were complex IV negative, no sequence analysis was possible (Figure 1). Combined defects of complex I and complex IV have also been described in other mitochondrial diseases, including MELAS (48) and MERRF (26) syndrome, both caused by mutations in mitochondrial tRNA genes.

4.4. Nasopharyngeal oncocytoma

Oncocytomas of the nasopharyngeal tract are extremely rare and have only been reported as single cases in the literature (49) (50). Previously, one case of a nasopharyngeal oncocytoma was described, which displayed a disruptive frame-shift mutation affecting the ND5 subunit of complex I (51). Associated loss of assembled complex I was demonstrated by immunohistochemistry and Western blot analysis. The disruptive *ND5* mutation was homoplasmic in the tumor nodule. In the patient, pre-existing heteroplasmy of 2% was found in blood cells and 9% in the minor salivary gland. Two siblings of the patient also carried the *ND5* mutation. One sibling had a heteroplasmy level of 12% mutated mtDNA in blood cells, whereas the other sibling carried only 2% mutated mtDNA in his leukocytes. Neither sibling developed cancer or any other disease. Three other siblings were deceased, one from cervical cancer, one from lung cancer, and the third from ischemic cardiopathy. Unfortunately, tissues from the deceased individuals were not available (51).

4.5. Salivary gland oncocytoma

Oncocytomas of the parotid gland account for <1% of salivary gland tumors and appear as oncocytic hyperplasia, benign oncocytoma, and rarely as oncocytic carcinoma (52) (53). Transcripts of mitochondrial genes in salivary gland oncocytomas were found to be increased up to 10-fold, which is in accordance with an observed 10-fold increase in mtDNA content. Accordingly, expression of nuclear genes involved in mitochondrial energy metabolism, ATP synthase subunit ß, and adenine nucleotide translocator isoform 2 (ANT2), was increased up to 30-fold in salivary gland oncocytomas, whereas only a 4-fold increase was observed in renal oncocytomas (23).

Here we report the analysis of 13 cases of salivary gland oncocytomas (mean age: 76 years; range: 63 - 89; 31% male). Ten cases of oncocytomas showed reduction of the complex I subunit NDUFS4 when
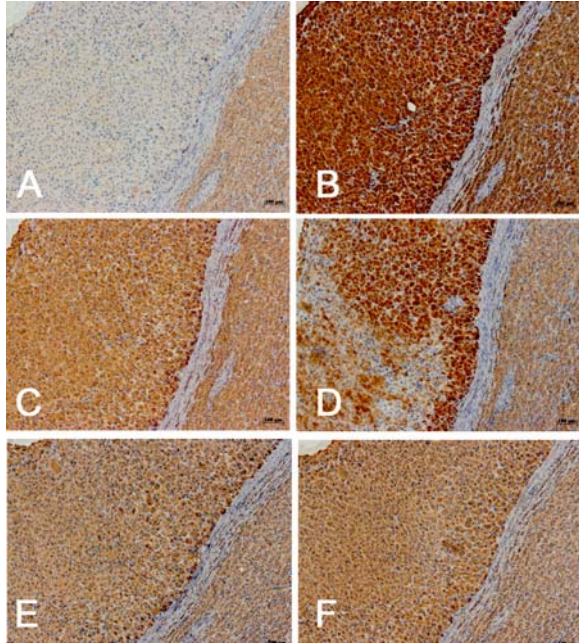
Figure 2. Immunohistochemical staining of all respiratory chain enzyme complexes and porin of an adrenal gland oncocytoma. Cells sare staining positive for complex I subunit NDUFS4 in normal tissue (right part) and negative in the corresponding oncocytic tumor tissue (left part) (A). Increased expression of respiratory chain complex II subunit 70 kDa (B), complex III subunit core 2 (C), and complex V subunit alpha (E) is present in the oncocytic tumor. Immunohistochemical staining of porin reveals the characteristic up-regulation of mitochondria in oncocytic tumor cells (F).

compared to the corresponding normal tissue (data not shown). Two cases exhibited positive staining for complex I and another case showed heterogeneous staining. In these cases assembly of complex I seemed to be unaffected. However, by immunohistochemical methods it is impossible to detect enzymatic activity of complex I. In these cases it is possible that the enzymatic activity but not the assembly of complex I could be affected. Complex II to V and porin were up-regulated in all tumor samples compared to adjacent normal tissue (data not shown). MtDNA mutations were found in the complex I genes *ND1*, *ND2*, *ND4* and *ND6* (Table 1). In total, five (38%) of the tumors contained mutations (3571_3572insC, 5107delG, 14368delT, 11403G>A; 11559G>A) in mtDNA which explain the absence of assembled complex I (Table 1); all mutations were found in the first third of the affected proteins. In eight cases (62%) no pathogenic mutations were found.

4.6. Adrenal gland oncocytoma

Oncocytic neoplasms of the adrenal gland are extremely rare (54) (55) (56). To our knowledge, no analysis of mitochondrial functionality in adrenal gland oncocytomas has been reported in the literature. Therefore, we examined one case of an adrenal gland oncocytoma (female, 51 years old). A specific lack of complex I was

also observed in this type of oncocytic tumor (Figure 2). As observed in all oncocytomas so far, up-regulation of OXPHOS complexes II to V and increased mitochondrial mass in the tumor sample were evident compared to the corresponding normal tissue (Figure 2). Sequence analysis revealed a deletion of one adenine in the *ND4* gene, causing a frame shift at Lys93 (Table 1).

4.7. Pituitary gland oncocytoma

Pituitary gland oncocytomas are regarded as variants of null cell adenomas, as they present the same clinical features but with additional mitochondrial mass (56) (57). Because oncocytic changes occur in null cell adenomas, pituitary gland oncocytomas are suggested to illustrate the end stage of null cell adenomas (58). Nishioka *et al.* examined eight cases of pituitary gland oncocytomas and found a heterogeneous composition of cells staining positive for a 65-kDa mitochondrial protein and/or subunit II of complex IV. Because the identity of the 65-kDa mitochondrial protein is not known, it is unclear if the antibody used to recognize it is an appropriate tool to estimate mitochondrial mass.

We searched for respiratory chain deficiencies in two cases of oncocytic pituitary gland adenomas (case 1: male, 59 years old; case 2: female, 72 years old). Both cases showed loss of complex I (data not shown). In addition, one case exhibited a complex IV deficiency in about 80% of the tumor cells. Respiratory chain complex II subunit 70 kDa, complex III subunit core 2, complex V subunit alpha, and the mitochondrial membrane protein porin, were all up-regulated. In one case, insertion of a cytosine between positions 11872 and 11873 was found in the *ND4* gene (Table 1). This mutation is located in the last third of the protein, causing a truncated ND4 subunit. In the second case, the one harboring the complex IV deficiency, additional mitochondrial tRNAs and complex IV subunits were sequenced but no pathogenic mutations were detected (data not shown).

4.8. Eyelid oncocytoma

Oncocytomas of the eyelid are extremely rare: only seven cases have been reported previously (59) and without any functional or genetic analysis of mitochondrial energy metabolism. For the present study only one case (female, 51 years old) was available for investigation of alterations of the respiratory chain complexes. As expected for an oncocytic tumor, a specific lack of complex I was also observed in the eyelid oncocytoma, and complex II, complex III, complex IV, complex V and porin all showed up-regulation, indicative of increased mitochondrial mass in the tumor sample compared to the corresponding normal tissue. Sequence analysis of mtDNA-encoded complex I subunits revealed no pathogenic mutation which could explain the loss of complex I.

4.9. Oncocytic tumors of other tissue

Mitochondrion-rich breast cancers are rare, although the prevalence of such tumors might be underestimated (60). One of five mitochondrion-rich breast tumors harbored a heteroplasmic disruptive mtDNA mutation and two cases had potentially pathogenic missense mutations (45) (Table 1).
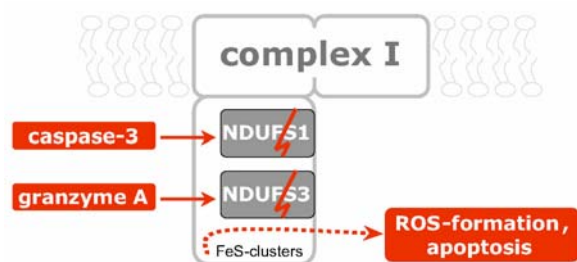
Figure 3. Protein subunits of complex I of the respiratory chain are a proteolytic target of either activated caspase-3 or granzyme A. Cleavage of subunit NDUFS1 or of NDUFS3 results in a loss of complex I activity and the formation of reactive oxygen species (ROS) by the iron-sulphur cluster part of the protein complex.

Oncocytic tumors of the pancreas (61), the stomach (62) and the meninges (63) are very rare and have usually been described only as single case reports. Therefore, such types of oncocytomas were not available for the present study and information regarding alterations of mitochondrial metabolism in such tumors is nonexistent.

5. DISCUSSION

Oncocytomas from eight different organs are now described as a tumor subgroup which is characterized by hyperproliferation of mitochondria in response to complex I deficiency. Renal, thyroid and nasopharyngeal oncocytomas were previously reported to lack complex I (27, 30-32, 45, 51). Onococytomas of the parathyroid, the salivary gland, the pituitary gland, the eyelid and the adrenal gland were first described as complex I-deficient in this paper. Consistent with their phenotype of complex I deficiency a high proportion of oncocytomas harbor somatic mtDNA mutations, most of them affecting complex I-encoding subunits. Interestingly, disruptive mutations of mtDNA-encoded complex I subunits were generally homoplasmic. This strongly suggests that mtDNA mutations are selected during the process of cell division, further indicating that mtDNA mutations that disrupt complex I activity exert a positive selection pressure on the cell. Because complex I deficiency has been detected in adenoma as well as in carcinoma, we hypothesize that the metabolic switch is an early event in oxyphilic tumor progression. This hypothesis is further supported by the fact that complex I deficiency is associated with tumor cell proliferation in an animal cancer model (64).

Different methods have been used in the literature to identify complex I defects in either frozen or formalin-fixed paraffin-embedded (FFPE) tissue sections. Measurement of enzymatic activity gives the best information on the functional status of energy metabolism, being independent from assembly status. However, this method depends on either fresh or snap-frozen tumor specimens, which are not routinely available in most centers. Another problem of enzymatic analysis is the cellular composition of the samples. Only homogenous

tumors such as oncocytomas of the kidney are appropriate for this kind of investigation because the analysis is performed using homogenized tissue lysates. Contamination with normal tissue will obscure the degree of an enzyme defect in a tumor. The use of paraffin-embedded tissues bypasses these problems as it permits the use of routinely fixed archival samples and analysis of respiratory defects at a single-cell level (27, 51). However, it has to be pointed out that this type of analysis only detects the loss of a protein and provides no information on the activity level in cases of detectable protein. Therefore, in the two cases of salivary gland oncocytomas, both with positive complex I staining, we cannot rule out that the enzymatic activity is lost.

In the past few years, evidence has emerged that complex I has a central role in apoptotic processes. It has been shown in cell culture experiments that the nuclear encoded complex I subunit NDUFS1 is disrupted by caspase-3 during staurosporine-induced apoptosis (65). Caspase-3 treatment turns complex I into a reactive oxygen species (ROS)-generating factor and causes loss of its NADH dehydrogenase activity. ROS production is followed by disruption of electron transport and transmembrane potential, loss of ATP production, and damage to mitochondria (Figure 3). Cells over-expressing a noncleavable variant of NDUFS1 maintained both transmembrane potential and ATP production, and ROS formation was not increased, mitochondrial morphology was unaffected, and plasma membrane integrity was stabilized (65). Therefore, loss of complex I could impair this apoptotic pathway, thereby giving complex I-deficient cells a survival advantage.

The combination of interferon-ß (IFN-ß) and retinoic-acid (RA) has synergistic effects on enhancing apoptosis and is used in cancer treatment. Application of IFN-ß/RA to a breast cancer cell line and to HeLa cells caused an up-regulation of various respiratory chain subunits, resulting in an increase of ROS production. Knockdown of the complex I subunit NDUFS3 or GRIM-19 in those same cell lines resulted in a reduction in complex I activity and less ROS production. Complex I knockdown cells showed increased survival and reduced apoptosis compared to wild-type cell lines (66). These data underline the fundamental role of complex I in cell death and its potential role in cancer treatment.

Another pathway that was shown to directly affect complex I involves granzyme A (GzmA) (67). Granzyme A is a serine protease released by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells to trigger apoptosis via a caspase-independent apoptotic pathway. GzmA enters the mitochondrial matrix and cleaves the nuclear encoded complex I subunit NDUFS3, which also results in generation of ROS, disruption of the transmembrane potential and ATP production, and production of DNA damage (Figure 3). Cells over-expressing an uncleavable variant of NDUFS3 showed reduced ROS production and protection from both transmembrane potential disruption and nuclear DNA fragmentation (67). Granzyme B (GzmB), which is is also

released by CTLs and NK cells, kills target cells by activation of caspase-3 (68). Due to the facts that GzmA directly targets NDUFS3 and GzmB activates caspase-3, which cleaves NDUFS1, we hypotesize that complex I-deficient cells might contribute to tumor-cell resistance to attacks by CTLs and NK cells.

A recent study by Ishikawa *et al.* (64)showed that mtDNA mutations causing complex I deficiency are associated with tumor cell metastasis. Cytoplasmic hybrids (cybrids) were used to exchange the mtDNA between mouse tumor cell lines having low and high metastatic potential. Cybrids with high metastatic potential harbored mutations in the mitochondrially encoded complex I subunit ND6, causing a loss of complex I activity, whereas cybrids with wild-type mtDNA and normal complex I activity had low metastatic potential (64).

Activation of an oncogene or inhibition of a tumor suppressor gene usually leads to a diversity of changes in intracellular signaling pathways and cellular metabolism. Therefore, loss of complex I might also have consequences for intermediary metabolites, leading to different intracellular adaptations of the tumor cells. Complex II impairment leads to accumulation of succinate. Complex I deficiency might also result in accumulation of upstream metabolites within the citric acid cycle, including succinate and fumarate. These compounds have been shown to be used by tumor cells to generate building blocks for amino acid and lipid synthesis (69). Furthermore, accumulation of succinate and fumarate promotes stabilization of HIF-1. HIF-1 is a transcription factor that up-regulates a number of genes involved in aerobic glycolysis (e.g. glucose transporters, glycolytic enzymes, lactate dehydrogenase) (70). Currently, information on the metabolic changes induced by complex I impairment in tumor cells is lacking. In patients with complex I deficiency, accumulation of lactate is observed. Elevation of lactate is also a common feature of most solid tumors with increased aerobic glycolysis and is thought to contribute to tumor metastasis (71).

The fact that tumors with impaired aerobic energy metabolism have to rely on glycolysis opens therapeutic options. In particular, in cases with a clear, specific impairment of the respiratory chain such as oncocytomas, inhibition of glycolysis or deprivation of glucose should be considered for therapy. Glycolytic inhibitors serve as a classic example of cancer metabolism-targeting agents (72). Another option to deprive tumor cells of energy production by glycolysis is through a ketogenic diet. This is a high-fat, low-carbohydrate diet, which switches the primary source of energy metabolism from glucose to ketone bodies. Generation of ketone bodies is a physiological adaption to prolonged food restriction. The use of ketone bodies for ATP production is strictly dependent on the aerobic mitochondrial energy metabolism. Normal cells can use either glucose or ketone bodies for energy metabolism, whereas complex I-deficient cells cannot use ketone bodies at all. The first use of a ketogenic diet occurred in 1995 in the treatment of human malignant brain cancers. Both patients responded well to the ketogenic diet and no further chemo- or radiation therapy was needed (73). Because cells with impaired OXPHOS depend on glucose as a primary energy source,

onocytic tumors, such as oncocytic thyroid carcinomas which have poor responsiveness to radioiodine, may be treated by a ketogenic diet or glycolysis inhibitors. This should even be effective in combination with classical tumor therapy.

In conclusion, data on different types of oncocytomas clearly demonstrate that impairment of respiratory chain complex I is a universal feature of oxyphilic tumors. Thus, oncocytoma could be classified as a mitochondrial disease. Given its potential role in cell proliferation and apoptosis, complex I can be considered a mitochondrial tumor suppressor.

6. ACKNOWLEDGMENT

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