

# Clonal expansion of mutated mitochondrial DNA is associated with tumor formation and complex I deficiency in the benign renal oncocytoma

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Mutations in mitochondrial DNA (mtDNA) are frequent in cancers but it is not yet clearly established whether they are modifier events involved in cancer progression or whether they are a consequence of tumorigenesis. Here we show a benign tumor type in which mtDNA mutations that lead to complex I (CI) enzyme deficiency are found in all tumors and are the only genetic alteration detected. Actually renal oncocytomas are homogeneous tumors characterized by dense accumulation of mitochondria and we had found that they are deficient in electron transport chain complex I (CI, NADH-ubiquinone oxidoreductase). In this work total sequencing of mtDNA showed that 9/9 tumors harbored point mutations in mtDNA, seven in CI genes, one in complex III, and one in the control region. 7/8 mutations were somatic. All tumors were somatically deficient for CI. The clonal amplification of mutated mtDNA in 8/9 tumors demonstrates that these alterations are selected and therefore favor or trigger growth. No nuclear DNA rearrangement was detected beside mtDNA defects. We hypothesize that functional deficiency of the oxidative phosphorylation CI could create a loop of amplification of mitochondria during cell division, impair substrates oxidation and increase intermediary metabolites availability.

## INTRODUCTION

Alterations of mitochondrial DNA (mtDNA) have been found in many types of cancers such as those of thyroid, breast, colon, ovary, liver, pancreas, prostate, lung, brain and gastric carcinoma (1–3). They are generally homoplasmic. Since they are not present in all tumors in a cancer type,

and since other well identified oncogenic events are also found beside, they are supposed to be modifier events. Hence the important debate is to determine if they are truly involved in tumor progression or if they are a consequence of it. Occurrence of more mutated mtDNA in tumors than in normal tissues provided evidence that these mutations are, at least, not deleterious for cancer cells. It is possible that they

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are a neutral consequence of tumor formation and that, after sufficient cell divisions, either mutant or normal mtDNA can be lost at random, producing a homoplasmic cell lineage (4). However, they could induce a loss of function and be advantageous to the cell proliferation. In this work we show a tumor type, renal oncocytomas, in which mtDNA mutations that lead to complex I (CI) enzyme deficiency are found in all tumors and are the only genetic alteration detected.

Oncocytomas are tumors characterized by dense accumulation of mitochondria. They are found in several epithelial tissues, from salivary and parathyroid glands to thyroid and kidney. Thyroid oncocytic tumors are heterogeneous tissues composed of more than 75% oncocytic cells, i.e. cells in which the dense mitochondrial granular network is easily stained by eosin. Thyroid carcinomas with oncocytic features are generally considered as more aggressive than other 'well-differentiated' thyroid cancers (5). Renal oncocytomas are essentially homogenous tumors with round cells which have lost their polarity and with regular, central nuclei (6). They are considered as benign in spite of rare cases of invasiveness and of mixed tumor types in the same kidney (6). *In vivo*, their growth rate is similar to that of the aggressive conventional renal carcinomas (7).

A mitochondrial defect had been suspected for a long time in oncocytomas since accumulation of abnormal mitochondria is also found in muscles of patients suffering from mitochondrial encephalomyopathies characterized by large deletions in mtDNA (8,9). Actually a deficiency of mitochondrial CI activity and protein contents has been demonstrated by our group in all tested renal oncocytomas (10). Conversely, enzyme activities of other oxidative phosphorylations (OXPHOS) complexes and of citrate synthase were increased, together with mtDNA content (11,12). CI or NADH-ubiquinone oxidoreductase is the main gateway to the electron transport chain. Hence, mitochondrial proliferation in oncocytic cells could be a regulatory response attempting to restore a defective respiratory function through a still unknown sensing mechanism. In agreement with this hypothesis, defective oxygen consumption has been shown in thyroid oncocytomas (13,14), while expression of PGC-1-related co-activator (PRC) that regulates mitochondrial proliferation was increased (15). More recently, homoplasmic, disruptive mtDNA mutations were found in CI genes in 26% of thyroid oncocytomas (3), demonstrating that the loss of function in CI was not deleterious for cancer cells and possibly a proliferative advantage (14).

To understand if CI deficiency observed in renal oncocytomas could be causative with respect to the tumor formation, one should first sequence the 45 structural genes of CI (4,16). CI is encoded in part by nuclear genome and in part by mitochondrial genome for seven genes (MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND4L, MT-ND5 and MT-ND6), yielding 45 subunits (17). Since several of these subunits are necessary for proper assembly of the complex, the lack of only one subunit, such as those encoded by MT-ND4 or MT-ND6 or GRIM19/NDUFA13 (nuclear DNA), or of an assembly factor may lead to degradation of all the remaining subunits (17,18). Such a general deficiency of all CI subunits was observed in renal oncocytomas (10) and it was therefore impossible to determine which subunit

could be genetically defective using a proteomic approach. Searches for large mtDNA deletions have shown no difference between normal and tumor tissues (6). Chromosomal abnormalities are frequent, yet not always present (6), and may affect several of the some 16 chromosomes harboring CI genes (Supplementary Material, Table S1).

In order to investigate genetic alterations of CI genes that would be specifically associated with tumors in renal oncocytomas, we combined several approaches. In some patients, nuclear genome abnormalities were investigated by comparative genomic hybridization (CGH) arrays, by karyotyping and by sequencing the cDNA of five CI subunits that are frequently mutated in familial mitochondrial diseases. Since no nuclear genome abnormality was found, mtDNA was totally sequenced. The results showed that mtDNA harbored disruptive point mutations in 8/9 tumors, 7 tumors with CI defects, and one with a defect in the mitochondrial gene of complex III (CIII) which is necessary for CI assembly (19). The last tumor carried a mutation in the control region. Mutations were mostly homoplasmic and disruptive. All tumors had a marked loss of CI enzyme function. Moreover, it could be demonstrated in two patients that no other abnormality was detectable in nuclear genome besides those of mitochondrial genome.

## RESULTS

### Point mutations were found in mtDNA from 9/9 renal oncocytomas essentially in complex I genes

The entire mtDNA sequence was obtained from nine renal oncocytomas and five high-grade clear cell renal cell carcinomas (CCRCCs). The results are summarized in Table 1. mtDNA point mutations were found in all oncocytomas and in none of the CCRCCs. Somatic origin of mutations was checked by sequencing the mutated region of mtDNA from adjacent normal tissue whenever available. The results showed that 7/8 tested oncocytomas were bearing tumor-specific mutations in mtDNA. Only one tumor harbored a non-somatic mutation [3571insC in patient 8, a mutation of ND1 already reported in two cases of thyroid oncocytoma (3)]. To estimate the potential loss of function, all mtDNA changes expected to result in defective protein product (nonsense and frame shift mutations) were classified as *disruptive* mutations. When the change resulted in an amino acid substitution, the putative damage was statistically estimated *in silico* with the Position-Specific Independent Counts (PSIC) score, calculated with the PolyPhen software (20), and mutations only predicted to be probably damaging by PolyPhen analysis were indicated as *potentially pathogenic*. One renal oncocytoma harbored a potentially pathogenic, homoplasmic missense mutation in MT-ND5, and seven out of nine oncocytic samples (77.8%) harbored disruptive mutations among which six were in CI subunits. Four such mutations generate a stop codon close to the N-terminus of the protein, likely leading to a more damaging effect on protein production. In one case a stop codon in MT-ND1 was generated by two close mutations, one germ-line variant (G3666A) not reported as polymorphic and one novel somatic mutation (G3664A). One disruptive mutation occurred in the cytochrome *b* gene

Table 1. mtDNA alterations found in renal tumors

Tumor type	Patient no.	Base change	AA change	Gene	% of mutated	PSIC	Already in database	Som	Term
Oncocytoma	1	<b>11872insC</b>	<b>374X</b>	ND4	100		Novel	+	C
Oncocytoma	1	G12923T	W196L	ND5	100	2.536	Novel	—	
Oncocytoma	2	<b>14429delG</b>	<b>87X</b>	ND6	100		Novel	+	N
Oncocytoma	3	T3308C	M1T	ND1	100	2.955	Mitomap	—	
Oncocytoma	3	<b>G3664A</b>	<b>G120X</b>	ND1	97.5		Novel	+	N
Oncocytoma	3	G3666A	<b>G120X</b>	ND1	100		HmtDB	—	
Oncocytoma	3	A12512T	Q59L	ND5	100	2.643	Novel	—	
Oncocytoma	3	C6567T	P222S	COI	38	2.604	Novel	+	
Oncocytoma	4	G564A	non coding	D-Loop	100		Mitomap	+	
Oncocytoma	5	<b>13937delAC</b>	<b>540X</b>	ND5	100		Novel	+	C
Oncocytoma	6	<b>G4063A</b>	<b>G244X</b>	ND1	95.5		Novel	+	C
Oncocytoma	7	T13847C	L504P	ND5	100	2.479	Novel	(ND)	
Oncocytoma	8	<b>3571insC</b>	<b>101X</b>	ND1	21.3		Mitomap	—	N
Oncocytoma	9	<b>15342insT</b>	<b>210X</b>	CYTB	100		Novel	+	N/C
Clear cell	10	No mutation in mtDNA							
Clear cell	11	No mutation in mtDNA							
Clear cell	12	No mutation in mtDNA							
Clear cell	13	No mutation in mtDNA							
Clear cell	14	No mutation in mtDNA							

Disruptive mutations are in bold. PSIC: probability of loss of function. Som: somatic alteration. Term: terminus from which the protein is expected to be truncated.

encoding a CIII subunit (patient 9). Sequencing the mtDNA from primary cultures of this tumor showed maintenance of the mutation in the culture conditions. Overall association between disruptive mutations and oncocytic phenotype was highly significant as tested by the Fisher’s exact test ( $P = 0.01$ ) and by the  $t$ -test for small samples ( $P = 0.0005$ ). The remaining tumor harbored a somatic mutation in the mtDNA control region (D-Loop). This mutation occurred in a conserved control region, the major H strand promoter, suggesting that it is important for mtDNA function.

Four oncocytic samples and one clear cell carcinoma presented novel mtDNA variants, reported in Supplementary Material, Table S2.

More than 95% mtDNA harbored the same mutation in tumor tissues

DNA regions harboring a mutation were cloned to estimate the percentage of altered mtDNA copies. With the exception of one case (3571insC in patient 8), tumor tissues were all essentially homoplasmic for their mutations. It should be added that in patient 3 a heteroplasmic mutation was found in MT-COI, a complex IV (CIV) gene, in addition to the two disruptive, homoplasmic mutations in MT-ND6 and MT-ND1 (Table 1).

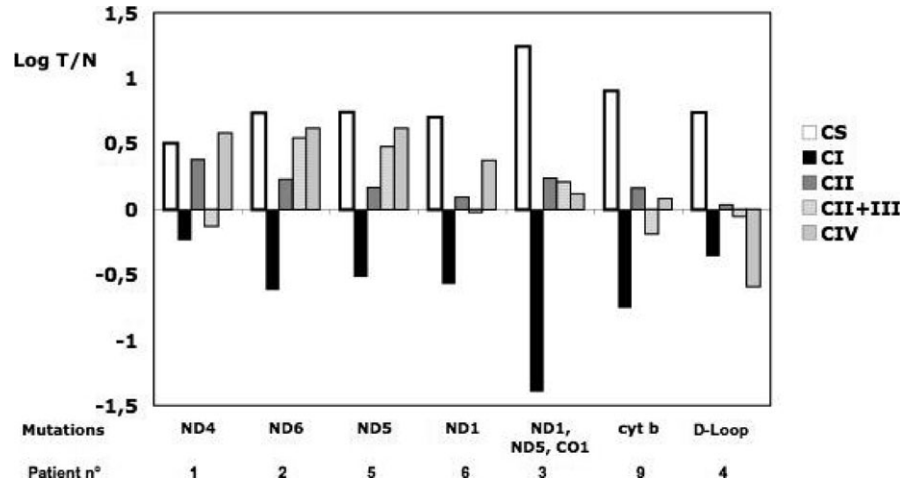
Mitochondrial DNA-synthesizing POLG gene was not defective

mtDNA is synthesized by polymerase  $\gamma$ , which is encoded by a nuclear gene (POLG). In order to exclude mutations in POLG as the cause for high occurrence of mtDNA mutations in oncocytic tissues, the coding region and the intron/exon boundaries of the POLG gene were entirely screened in all 14 cases analyzed for mtDNA sequencing. All variants found were already reported SNPs with high frequency of

occurrence in the general population (data not shown), except for one novel missense variant (G5138C) in patient 7 whose somaticity could not be tested due to lack of the corresponding normal tissue. *In silico* prediction of the amino acid change (Q226H) indicated the non-pathogenic potential of the variant (PSIC = 0.117).

No nuclear genome abnormality could be detected in the tested tumors

Mutations were searched in the coding sequence of five CI subunits that are frequently altered in familial mitochondriopathies: *NDUFS1* (75 kDa), *NDUFS2* (49 kDa), *NDUFS4* (18 kDa), *NDUFS7* (PSST), *NDUFS8* (TYKY), and *NDUFV1* (51 kDa). No mutation could be found in tumor or normal tissues from patients 1, 2 and 5. Nuclear DNA rearrangements or amplifications were then investigated by CGH array in patient 2. Normal or tumor DNA were hybridized on an oligonucleotide 44 k microarray slide (Agilent), in competition with a normal DNA control. Two slides were used for each tissue, with alternative dual-color competition in order to crosscheck variations. No genomic DNA rearrangement was found in tumor tissue, nor in normal adjacent tissue. In conclusion, the mtDNA defect of ND6 was the only genetic event detected in this tumor by our techniques. Since the tumor from patient 4 harbored an mtDNA mutation in a non-coding region, its pathogenicity could not be ascertained. We investigated nuclear DNA rearrangements and mutations in *BHD*, a gene which occurs to be altered in oncocytomas (21) from Birt-Hogg-Dubé syndrome. Chromosome analysis showed a normal karyotype. We demonstrated no anomalies except non clonal monosomies 3 and 7, 8 and 18 in normal cells and non-clonal monosomy 18 in tumor cells. Fluorescence *in situ* hybridization (FISH) analysis of 11q13 breakpoint region that is sometimes altered in oncocytomas



**Figure 1.** Increased citrate synthase and decreased complex I activities are hallmarks of renal oncocytomas. Enzyme activities of citrate synthase (CS), complex I (CI), complex II (CII), complexes II+III (CII+III) and complex IV (CIV) were determined in paired tissues from seven patients, expressed in log (tumor activity/paired normal tissue activity), and the results aligned with the genetic defects according to Table 1. Complex I was decreased in all oncocytomas, including those from patients 9 (mutated in cytochrome *b*, complex III subunit) and 4 (mutated in control region). In patient 4 all complexes were decreased, except CII which is totally encoded by nuclear genome. Citrate synthase was increased in all oncocytomas. CIII was sometimes decreased, particularly in the cytochrome *b* deficient tumor from patient 9. CIV was decreased in patient 4.

(22) focused the PRAD1 region in two cultures. The relative positions of CCND1 signals were normal. There was no mutation in the *BHD* gene.

#### Citrate synthase activity always increased, NADH dehydrogenase activity always decreased in tumor tissues

In order to understand the potential effect of mutations on mitochondrial function, we determined in each tumor the enzyme activities of citrate synthase and of respiratory chain complexes I (NADH coenzyme Q oxidoreductase), II (succinate dehydrogenase), II+III (succinate-cytochrome *c* dehydrogenase) and IV (cytochrome *c* oxidase). As shown in Figure 1, citrate synthase activity was increased in all tumors, whatever was the mtDNA mutation. This mitochondrial matrix enzyme is generally accepted to reflect mitochondrial content, whereas OXPHOS activities have been shown to vary to some extent independently from mitochondrial density (4). Figure 1 shows that CI was inhibited in all tumors, including those harboring mutations in CIII and in the D-Loop (Fig. 1). Indeed in patient 9, cytochrome *b* disruptive mutation was associated with loss of activities in CIII and CI, and in patient 4, a mutation in the control region was associated with an overall loss of activity of all tested OXPHOS complexes that are encoded by mtDNA, particularly CI. CIV (cytochrome *c* oxidase) increase has been proposed as a marker of renal oncocytomas (11). However, Figure 1 shows that CIV may be unchanged under the influence of a heteroplasmic mutation in one of its genes (patient 3) or decreased (patient 4), without hindering the oncocyctic phenotype. Therefore, as previously shown (10) CI deficiency and citrate synthase increase are the most specific features of renal oncocytomas.

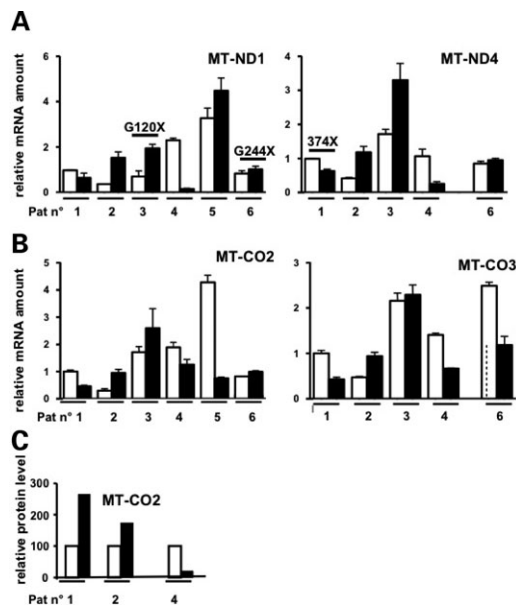
#### OXPHOS subunit levels were not regulated through their mRNA levels

From previous studies confirmed in this work, the amounts of OXPHOS protein subunits and OXPHOS enzyme activities have been shown to be increased in oncocytomas, except for CI, the subunits of which were under-expressed (10). In the search for a link between mtDNA mutations and their functional consequences, transcripts encoded by the mitochondrial genome were determined by RT-PCR. As shown in Figure 2, transcripts from mutated genes were in some cases over-expressed (patient 3) or under-expressed (patient 1). Non-defective transcript expressions paralleled those of defective mRNAs in each individual tumor (Fig. 2), yet their levels were by no means correlated to the loss of protein and function of CI, nor to the gain of protein and function of CIV. Therefore the levels of CI protein subunits depended on a translational event for those bearing disruptive mutations and from a post-translational event for the remnant OXPHOS protein subunits. At variance, all transcripts from mtDNA harboring a mutation in the major H strand promoter (patient 4) were decreased, suggesting that the general decrease of OXPHOS enzymes in this tumor is linked to an mtDNA transcriptional impairment.

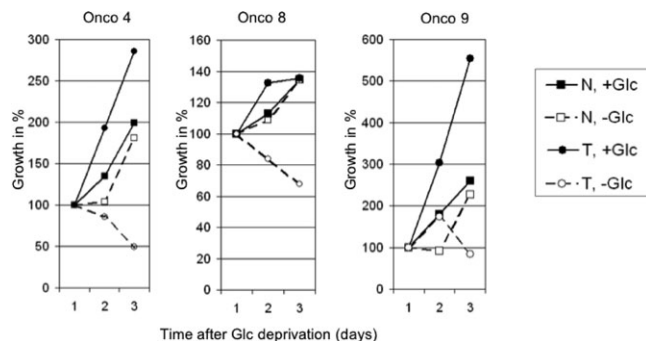
#### Oncocytoma cells in primary culture were addicted to glucose for their survival and growth

In order to estimate the metabolic changes occurring in oncocytomas, freshly sampled tissue from oncocytomas and from the adjacent normal tissue were digested to isolated tubular cells which were then grown in the presence of the best respiration substrates for kidney, i.e. ketone bodies (23). Figure 3 shows that when the normal cells were deprived of glucose, their growth could be maintained by ketone bodies, whereas oncocyctic cells from several oncocytomas were all addicted to glucose for their survival and growth.





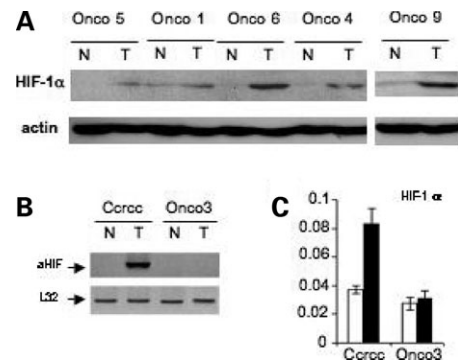
**Figure 2.** Mitochondrially encoded oxidative phosphorylations (OXPHOS) subunits are not regulated though their mRNA levels in mitochondrial DNA (mtDNA) deficient tumors. Expressions of four genes encoded by mtDNA (MT-ND1, MT-ND4, MT-CO<sub>2</sub>, MT-CO<sub>3</sub>) were compared by qRT-PCR in paired normal (white bars) and tumor (black bar) tissues of 6 patients. mRNA levels are shown in (A) and (B). (A) Complex I (CI) subunits; mutated genes are indicated by a line and the amino acid change above the bars. Mutations may result in increased levels of all mRNAs, including that encoded by the mutated gene (patient 3) or decrease of all mRNAs (patient 1). (B) Complex IV (CIV) subunits; transcript levels changes grossly parallel those of CI transcripts in each patient. (C) Protein level of a CIV subunit; the protein level in tumor from patient 1 was increased in spite of the decreased level of its mRNA, and paralleled the enzyme activity shown in Figure 1. At the opposite, the mutation in control region (tumor from patient 4) was associated with a general decrease of mitochondrial transcripts and of one tested corresponding protein.



**Figure 3.** Oncocytoma cells were addicted to glucose for their growth. Cells isolated from tumor tissue (T) and its normal counterpart (N) were freshly cultured (patient 8, Onco 8) or passed twice (Onco 4 and 9). After mid-confluence, the DMEM/F12 medium contained either 5 mM glucose (+glc) or 10 mM beta OH-butyrate+3 mM acetoacetate (-glc). Growth was estimated by protein content. Patient numbers are the same as in Table 1. Glucose addiction in the presence of ketone bodies shows the loss of capacity to sustain growth with a respiratory fuel.

**HIF1 $\alpha$  protein was over-expressed in oncocytomas**

Two familial mitochondrial defects have been shown to favor tumor formation in several organs including kidney, namely



**Figure 4.** HIF1 $\alpha$  protein level was increased in renal oncocytomas. (A) HIF1 $\alpha$  protein was determined by immunoblotting in total tissue extracts (tumor: T, normal: N). Patient numbers are as in Table 1. (B) and (C), transcript levels of the HIF system in oncocytoma and a control clear cell renal cell carcinoma (CCRCC). (B) In oncocytoma (Onco 3, patient 3) the natural antisense transcript aHIF was absent. (C) HIF1 $\alpha$  mRNA level was not changed in tumor from patient 9 (qRT-PCR normalized to L32 mRNA level), whereas its protein level was increased as shown above.

those of fumarate hydratase (FH) and of genes encoding subunits of CII/succinate dehydrogenase (SDHD, SDHB and, to a lesser extent, SDHC) (21,24,25). The tumors they cause over-express the hypoxia inducible factor subunit HIF1 $\alpha$ . We therefore tested if this transcription factor was recruited in renal oncocytomas. Figure 4 shows that HIF1 $\alpha$  protein amount was mildly increased in oncocytomas, whereas its mRNA level did not change, nor did aHIF, the natural antisense transcript of HIF1 $\alpha$ . In contrast, HIF1 $\alpha$  and aHIF RNA levels were increased in a control clear cell carcinoma, as already shown by Thrash-Bingham and Tartof (26).

**DISCUSSION**

In the present work, we describe for the first time a pure type of homogenous benign tumor that is almost always associated with homoplasmic mtDNA defects and with mitochondrial proliferation, therefore exhibiting a triple clonal expansion of mutated mtDNA, mitochondria and tumor cells.

**mtDNA mutations and complex I inhibition**

This work shows that, consistent with their phenotype of CI deficiency, most renal oncocytomas harbor somatic mutations in genes encoding CI subunits. In two out of nine tumors, the mtDNA defect was localized outside CI genes, namely in cytochrome b gene and in the control region, yet in these tumors CI was also defective. Mutations in cytochrome b, the only CIII subunit encoded by mtDNA, may in fact induce a CI deficiency, because respiratory complexes are bound into supercomplexes or ‘respirasomes’ in the inner mitochondrial membrane. Absence of the cytochrome b protein may cause the dissociation of the whole supercomplex and hence the degradation of CI-free subunits (19). The G564A mutation in the non-coding mtDNA control region (major H strand promoter) had already been reported in Mitomap (URL) and was found in a patient suffering from progressive external ophthalmoplegia (PEO). The functional

consequence of this mutation was unknown at that time, however, the fact that, in the present study, it was associated with an overall under-expression of all mitochondrially encoded transcripts strongly suggests that it induces a severe impairment of mtDNA transcription. It should be underlined that this tumor harbored no mutation in POLG, which is frequently mutated in PEO, nor chromosomal rearrangement. Taken as a whole, mtDNA somatic mutations in renal oncocyoma are found mainly in CI genes, that cover the majority of mtDNA sequence, and in regions that may indirectly cause a CI deficiency, such as in cytochrome *b* and in the control region. No nuclear alteration was found in CI genes in this study, nor in other chromosomal regions. They cannot be totally ruled out since point mutations may remain undetected with our techniques. Nevertheless, mtDNA mutations that are enough to explain CI deficiency were found in all tumors.

### Complex I alterations in oncocytomas and mitochondrial accumulation

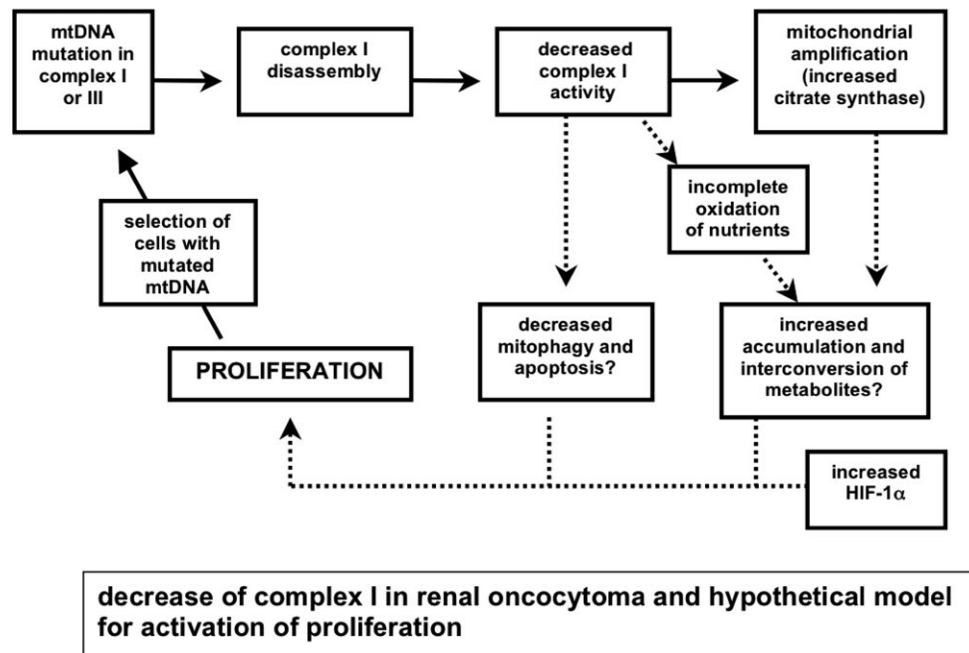
The present study shows that in the benign renal oncocytoma, accumulation of mitochondria is exclusively associated with mutations that induce a loss of CI or of CI and CIII, suggesting that mitochondrial accumulation is linked to altered function of complexes producing reactive oxygen species (ROS). In agreement, alterations of mitochondrial CI and CIII genes and of one nuclear CI gene, GRIM19/NDUFA13, have been found respectively in 26% and in 15% of thyroid oncocytomas (3,27) which on the contrary may occur often under malignant forms. Symmetrically, mutations in mtDNA encoding CIV in 11–12% of prostate cancers are not associated with mitochondrial proliferation (28). Mitochondrial accumulation in oncocytomas could be caused by increased biogenesis or decreased mitophagy (or both). Indeed, in non proliferating tissues, mitochondria divide and are eliminated (4) with a turnover half-life that is particularly short in kidney (6–10 days) (29). They are degraded by mitochondria-specific autophagy termed mitophagy. Mitochondria that harbor mtDNA mutations could be selectively eliminated because they produce too much ROS which alter the conformation of mitochondrial external membrane proteins (30). Alternatively they could be selectively retained because a defect in ROS-producing CI or CIII decreases the membrane signal for mitochondria elimination and, further, the apoptotic process (30,31). We show here that the general counter regulation of non-defective complexes is not driven by increased mtDNA transcription, but occurs downstream of translation. This supports the hypothesis that oncocyctic accumulation of mitochondria may result from a defect in mitophagy. In agreement, other authors have described intermediate oncocytomas with prominent intracytoplasmic vacuoles that originate from mitochondria (32).

### Complex I-invalidating mutations, mitochondrial accumulation and cell proliferation

The most fascinating feature of renal oncocytomas is that they associate mtDNA defects with proliferation. Nearly all mutations were somatic, associated with a tumor-specific loss of OXPHOS function and were homoplasmic. This

strongly suggests that mutated mtDNA is selected during the process of cell division, and therefore during the complex associated processes of mitochondria division, partition, and/or elimination, and of mtDNA partition between mitochondria and, beyond mitochondria, cells. In other words, the present study strongly suggests that mtDNA mutations that alter CI enzyme activity exert a positive selection pressure on the cells. In addition, the failure to find any chromosomal rearrangement in two tumors in which the mtDNA defect is well identified and linked to a CI loss of function strongly suggests that CI deficiency may induce proliferation in kidney. Concerning the putative mechanism of oncocytoma formation, we hypothesize that a loop of amplification may be created during the observed process of mutated mtDNA selection, as shown in Figure 5. An indirect link between CI and proliferation had previously been established in cancer cell lines. Inactivations of NDUFS13/GRIM19 and of NDUFA3, two nucleus-encoded CI genes, are able to prevent apoptosis induced by retinoic acid plus interferon (27). Similarly, it has been shown that rotenone inhibition of CI decreases ROS level and ROS-induced apoptosis in hepatoma cells (33). Such a mechanism could provide an explanation for the antiparallel changes of citrate synthase and CI activities in renal oncocytomas, through defective mitophagy, though the link between this paradoxical defect and tumor formation is not yet clear. Renal oncocytomas are benign but proliferating tumors and hence the question is to know if mtDNA mutations can be involved as modifier events in malignant cancers. In fact it was recently shown that CI disruptive mutations in mtDNA were found preferentially in oncocyctic thyroid tumors that are considered as more aggressive than other well-differentiated thyroid cancers (3). Altogether this suggests that alterations in OXPHOS function are true modifiers of carcinogenesis.

In this work, no mtDNA mutation could be found in five clear cell nephrocarcinomas, however, it should be mentioned that in these aggressive tumors OXPHOS activity is even more severely depressed than in oncocytomas due to the deficiency of *vhl* gene (12,34,35). In low grade CCRCCs, citrate synthase activity is decreased, but in high grades, citrate synthase and mtDNA levels are less depressed, probably under the influence of additional genetic events (36), resulting in OXPHOS defective cells with a granular cytoplasm reflecting a partially restored mitochondrial density. Therefore, decreased OXPHOS and maintained citrate synthase are correlated with aggressiveness in renal cancer, probably due to the need to redirect two-carbon compounds toward biosyntheses. Decreased synthesis of ATP by OXPHOS is compensated by glycolysis in all tumors, and, in agreement, we found in this study that renal oncocytomas strictly depend on glucose provision to grow. It was shown in hepatoma cancer cells that the citrate cycle is truncated, becoming a 'citrate pathway'. Citrate is formed in mitochondria from glutamine and 2-oxoglutarate and redirected to cytoplasm to feed lipid biosyntheses (37). Accordingly, inhibition of cytoplasmic acetyl-CoA formation from citrate in a cancer cell line strongly limits proliferation and survival (38). In renal oncocytomas, associated CI decrease and mitochondrial matrix activities increase could result in increased interconversion of precursor metabolites by intermediary metabolism and increased



**Figure 5.** Hypothetical model for feedforward amplification of proliferation in renal oncocytomas. Plain arrows represent reasonably established links, dotted arrows are hypotheses.

availability of these precursors for biosyntheses (Fig. 5). However, if this metabolic change is prone to favor growth, it is unclear how, *per se*, it could trigger the coordinate events required for re-entry into the cell cycle. Indeed, kidney tubule is considered as essentially quiescent, i.e. devoid of mitosis and of apoptosis, yet it has been recently shown to be able to divide without breaking the epithelial barrier in a very small fraction of cells (39). In addition, kidney tubule presents a powerful capacity to re-enter the cell cycle during regeneration, and, doing so, to reactivate both mitosis and apoptosis (40).

Intermediary metabolite levels could be involved in the signaling for cell growth as put forward by the works of Gottlieb and co-workers (41). Indeed other genes encoding mitochondrial enzymes, those of FH and of succinate dehydrogenase, have been shown to be genuine tumor suppressors. In the tumors they cause, there is accumulation of succinate (42) which is the product of HIF-prolyl hydroxylase and which inhibits HIF1 $\alpha$  degradation by a negative feedback mechanism. In agreement, HIF1 $\alpha$  is increased in cells supplied with succinate (41), demonstrating that succinate increase is able to recruit a transcription factor widely involved in cell metabolism and survival. The results of the present study show that a third mitochondrial defect, that of CI, is associated with increased HIF1 $\alpha$  at the protein level. Whether other pivotal regulators are stimulated to up regulate cell cycle rate in renal oncocytomas is now in progress.

To summarize, this work has shown that the key biochemical feature of renal oncocytoma is the association of increased citrate synthase and decreased CI activity, and that this phenotype is caused by mutations in mtDNA that lead to loss of CI protein subunits (Fig. 5). Proliferation in mutated cells is initiated by an unknown mechanism and mutated mtDNA is selectively concentrated to homoplasmy, showing that the

functional alteration is beneficial for cell growth (Fig. 5). These cumulated processes result in a feedforward loop of amplification for mutated mtDNA, altered mitochondria and altered cells (Fig. 5). We hypothesize that decreased CI enzyme activity could decrease mitophagy and apoptosis, and that accumulation of intermediary metabolites could favor growth or trigger cell cycle re-entry (Fig. 5).

In conclusion, this study has demonstrated that the benign renal oncocytomas are most interesting tools to investigate mitochondrial biogenesis and its relationship with tumor formation. Indeed, several recent discoveries have demonstrated the importance of concomitant OXPHOS decrease and mitochondrial biogenesis in cancer [reviewed in Gordan *et al.* (43) and Sutphin *et al.* (44)]. The study of renal oncocytomas should provide clues to unravel the functional meaning of these changes.

## METHODS

### Tumor sampling and diagnosis

All patients were informed of the anonymous use of their tissues for research and consented. Kidneys were stored on ice up to 1 h after removal and samples of tumor and cortical normal tissues were rapidly frozen in liquid nitrogen and stored in a tumor tissue bank (Tumorotheque des Hospices Civils de Lyon, DHOS-INCa). For diagnosis, fragments were formalin-fixed and paraffin-embedded, then stained with HES and Hale's colloidal iron stain. The diagnosis was made on: (a) presence of compact nests and/or of acinar, tubular or microcystic patterns, (b) round to polygonal cells with densely granular eosinophilic cytoplasm, round and regular nuclei with a centrally placed nucleolus; (c) presence of intra-cytoplasmic, apically located iron deposits.



### mtDNA sequencing

Frozen tissues (10–20 mg) were ground in a micro glass–glass Potter-Elvehjem homogenizer and total DNA was extracted using the GenElute™ kit for mammalian genomic DNA (Sigma, France). mtDNA from all samples was entirely sequenced using the MitoAll kit (Applera) according to manufacturer's instructions as previously described (3) and genomic sequences submitted to the HmtDB website.

### Heteroplasmy evaluation

Heteroplasmic status of disruptive mutations and potentially pathogenic changes was evaluated by cloning as previously described (3). At least 50 colonies were screened in order to calculate the percentage of mutated copies.

### Nuclear DNA alterations

**POLG mutations screening.** *POLG* was amplified in tumor samples using 24 primer pairs with different amplification conditions. Primers were designed within introns in order to cover whole exons and regulatory splice site regions, using the program Primer3 ([http://www.genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)). Direct sequencing was carried out on both strands with Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) according to standard manufacturer's protocol, on a ABI Prism 3730 DNA Analyzer (Applied Biosystems) and data analyzed using Sequencher, version 4.7. Sequence were compared to public SNPs databases and the software PolyPhen was used to calculate PSIC scores as previously described.

**Coding sequences of complex I nuclear genes.** Frozen tissues were ground in lysis buffer using a Polytron®. Total RNA was extracted with Trizol™ reagent (Invitrogen, France), then cDNA was synthesized and sequenced as previously described as follows: *NDUFS2* in Loeffen *et al.* (45), *NDUFS4* in van den Heuven *et al.* (46), *NDUFS7* in Triepels *et al.* (47), *NDUFS8* in Loeffen *et al.* (48), *NDUFV1* in Schuelke *et al.* (49). *NDUFS1* was sequenced with 4 PCRs (1, FOR: CAG ACA GTT TAG CAG AAC AG, REV: CCC AAA TCA TCT ACT CCT GC, 58°C, 634 bp, 2, FOR: GTT TGG AAA TGA TAG GAG CCG, REV: AAA CGT GGG TTT GTA CCA ACC, 58°C, 793 bp, 3, FOR: GTG GAC TCT GAC ACC TTA AG, REV: CTC TAC CCT CAG TGT TGA C, 58°C, 726 bp, 4, FOR: AGA TGG AGG TTG TAT CAC ACG, REV: GGA TCA CTG CAC TAC AGT TG, 58°C, 597 bp).

*BHD* gene was sequenced as described before (50).

**Comparative genomic hybridization.** Total DNA was extracted as described above and 500 ng DNA from paired normal and tumoral tissue as well as from reference DNA (Promega) were digested with AluI and Rsa I. Each sample (including reference) was separated in two aliquots that were labeled with Cy5 or Cy3 dUTP dyes, Klenow fragment and random primers. Reagents and protocol instructions were from Agilent CGH Labeling kit. Each labeled experimental DNA was mixed with the reference DNA carrying a different

label, providing four combinations, and each mix was hybridized on a Agilent Human Genome CGH Microarrays slides of 44 k containing *in situ* synthesized 60-mer oligonucleotides. After washing, slides were scanned on an Agilent 2565BA microarray scanner. Images were analyzed with Feature Extraction CGH Analytics softwares from Agilent Technologies.

**Chromosomal analysis.** Tumor cells were prepared from patient 4 and cultured as described below. After stimulation with phytohemagglutinin A, chromosomes from cells in metaphase were examined using R-banding (RHG) at 400 band resolution.

**Molecular cytogenetics.** FISH analysis was performed on cultured normal and tumoral renal cells in metaphase using probes D11Z1 and CCND1 for the 11q13 region (clone cSRL 129F9, Abbott Inc.).

### Enzyme activities

Frozen tissues (30–100 mg) were ground in Tris buffer containing protease inhibitors as previously described and immediately used for all enzymes determination (12). The most fragile, CI, was determined first as previously described (10). Each measurement was tested with bovine mitochondria as a positive control, performed in at least three different tissue dilutions to ensure proportionality and normalized to protein content estimated with the Bradford reagent.

### mRNA determination

Frozen tissues were ground in lysis buffer using a Polytron®. Total RNA was extracted with Trizol™ reagent (Invitrogen, France) and retrotranscribed to double stranded cDNA as previously described (51). All mRNAs were standardized with the ribosomal protein L32 mRNA. Semi-quantitative PCRs: a 100 µl PCR mix was distributed in several tubes and reactions were stopped with an interval of three cycles. Hybridization T° and primers were as follows: aHIF, 50°C, FOR: TTTGTG TTTGAGCATTTTAATAGGC and REV: CCAGGCCCTTT GATCAGCTT, MT-ND1, 55°C, FOR ACCAAGACCCTACT TCTAAC, REV: TTTTTTTTTTTTTTTTTTTTTTTT TAGG TT, MT-ND4, 55°C, FOR: GTGCTAAGTAACCACGTT CTC, REV: TTTTTTTTTTTTTTTTTTTTTTTT AAGAG, MT-CO<sub>2</sub>, 62°C, FOR: CACTGCRGTCCCCACA and REV: TTTTTTTTTTTTTTTTTTTTTTTT CTATAG, MT-CO<sub>3</sub>: 62°C, FOR: GTAGCCACAGGCTTCCACGG and REV: TT TTTTTTTTTTTTTTTTTTTTTTTT AAGACC. Quantitative PCRs were performed in a LightCycler instrument, in the presence of SYBR Green™ (Roche Diagnostics) using the following specifications: HIF1α, 65°C, FOR: CTGGGACA GCTTACCAAAACAGAGC, REV: TTAAGTTGATCCAA AGCTCTGAG, L32: 60°C, FOR: GTGAAGCCGAAGATC and REV: TTGTTGCACATCAGCAGC. All DNA contents were normalized using the control transcript L32.



### Immunoblotting

Total proteins of frozen tissues were solubilized, separated on 10% polyacrylamide gels and blotted as described previously (34). HIF1 $\alpha$  protein was detected with the Abcam polyclonal antibody (1/250) and actin with the monoclonal antibody from Chemicon (1/1000).

### Primary cell culture

Fresh tissues (normal and tumor) were saved in HEH2 organ conservation medium (IGL, Saint-Didier-au-Mont-d'Or, France) up to 24 h. Tissues were digested as previously described (51) in DMEM/F12 medium with 20 mg collagenase, 25 mg BSA and 100  $\mu$ l DNase I (10  $\mu$ g/ $\mu$ l) (Sigma) at 37°C with 75% air/5% CO<sub>2</sub>. The gross tubule preparation was then filtered through a 200  $\mu$ m gauze filter to eliminate glomeruli and totally digested into separate cells with 25 mg of BSA, 20 mg collagenase, 100  $\mu$ l DNaseI, 15 mg hyaluronidase and 50  $\mu$ l trypsin inhibitor (20 g/L) (Sigma). Cells were washed and resuspended in RPMI 1640 medium supplemented with 10% FCS (Invitrogen), penicillin-streptomycin (100  $\mu$ g/ml), amphotericin B (2.5  $\mu$ g/ml) (Sigma) for cell culture. Sodium pyruvate (5 mM) and uridine (50  $\mu$ g/ml) were added to tumor cell medium when passages were needed, as generally used in cells cultured from mitochondriopathies.

For glucose dependence studies RPMI medium was replaced by DMEM/F12 containing either 1 g/l of D-glucose or 3 mM of lithium acetoacetate plus 10 mM beta-hydroxybutyrate (Sigma, France). Growth was estimated by protein content with the Bradford Assay (BioRad, France).

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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