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Low mitochondrial respiratory chain content correlates with tumor aggressiveness in renal cell carcinoma

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A mechanism decreasing oxidative metabolism during normal cell division and growth is expected to direct substrates toward biosyntheses rather than toward complete oxidation to CO_2. Hence, any event decreasing oxidative phosphorylations (OXPHOS) could provide a proliferating advantage to a transformed or tumor cell in an oxidative tissue. To test this hypothesis, we studied mitochondrial enzymes, DNA and OXPHOS protein content in three types of renal tumors from 25 patients. Renal cell carcinomas (RCCs) of clear cell type (CCRCCs) originate from the proximal tubule and are most aggressive. Chromophilic RCCs, from similar proximal origin, are less aggressive. The benign renal oncocytomas originate from collecting duct cells.

Mitochondrial enzyme and DNA contents in all tumor types or grades differed significantly from normal tissue. Mitochondrial impairment increased from the less aggressive to the most aggressive RCCs, and correlated with a considerably decreased content of OXPHOS complexes (complexes II, III, and IV of the respiratory chain, and ATPase/ATP synthase) rather than to the mitochondrial content (citrate synthase and mitochondrial (mt)DNA). In benign oncocytoma, some mitochondrial parameters (mtDNA, citrate synthase, and complex IV) were increased 4- to 7-fold, and some were slightly increased by a factor of 2 (complex V) or close to normal (complexes II and III).

A low content of complex V protein was found in all CCRCC and chromophilic tumors studied. However F_1-ATPase activity was not consistently decreased and its impairment was associated with increased aggressiveness in CCRCCs. Immunodetection of free F_1-sector of complex V demonstrated a disturbed assembly/stability of complex V in several CCRCC and chromophilic tumors.

All results are in agreement with the hypothesis that a decreased OXPHOS capacity favors faster growth or increased invasiveness.

Abbreviations: RCCs, renal cell carcinomas; CCRCCs, clear cell type renal cell carcinomas; OXPHOS, oxidative phosphorylations; VHL, von Hippel–Lindau.

Introduction

Interest in the relationship between energy metabolism and tumorigenesis has been renewed recently by the discovery that the von Hippel–Lindau (VHL) tumor suppressor protein is regulating the oxygen-dependent gene expression (1–3).

The high rate of the Embden–Meyerhof glycolytic pathway in tumoral and transformed cells has been known for decades (4,5). Dang and Semenza (2) hypothesized that tumorigenesis is only possible when cell growth modification is associated with an increased expression of genes involved in the anaerobic glycolytic pathway. This would be necessary for the cell to survive to hypoxia caused by the absence of vascularization in the beginning solid tumor. However Vaupel *et al.* (4) noticed that, in ischemia, hypoxia occurs together with a decreased glucose provision, limiting the putative advantage of anaerobic glycolysis. On the other hand, a low oxidative metabolism could be a physiological status for fast-growing cells. Indeed, lymphocytes, enterocytes and fetal tissues are poorly oxidative (6,7) whereas highly oxidative tissues such as kidney cortex or brain are normally quiescent. In addition, hypoxia and high glucose were recently shown to exaggerate renal mesangial cell growth (8). Therefore a regulatory mechanism might exist which decreases oxidative metabolism in response to cell cycle regulators and then directs substrates toward biosyntheses rather than toward complete oxidation to CO_2. Conversely, high oxygen provision to some tissues could inhibit mitosis, maybe through the same biological switch. Several factors, among which the von Hippel–Lindau protein (1,9–11), could modulate the Embden–Meyerhof pathway and the oxidative phosphorylations (OXPHOS) in a cell cycle dependent way. However, any event invalidating the respiratory chain, either issuing from genetic damage or from the cell environment (12) could provide a proliferating advantage to a transformed or tumoral cell. We therefore asked whether there is a general correlation between reduced oxidative phosphorylation and aggressiveness in several renal tumor types.

Until now, there have been few studies related to the respiratory chain activity in tumors. Most of the available data have been obtained *in vitro* from the experimentally, chemically induced Morris hepatomas, but the genetic alterations associated with these different types of liver cancer only begin to be identified (13). Some tumoral cell lines may use oxygen, whereas some others are not able to do so (5). Hepatoma cell lines presenting with high glycolytic rate and decreased mitochondrial density are correlated with the highest growth rate (4,5,7,14).

Renal tumors originate from different nephron parts and are due to variable, but relatively characterized genetic events. Similarly their histological features and their aggressiveness are variable (15). More than 70% of renal cell carcinomas (RCCs) are the so-called conventional or clear cell carcinomas (CCRCCs), one of the most aggressive RCCs (15,16). They originate from the proximal tubule (15,17), a highly oxygen consuming cell type (18). They are characterized by a cyto-

plasmic accumulation of lipids and glycogen (15,19), an increase of enzymes from the Embden–Meyerhof pathway, and decreased gluconeogenic enzyme activities (19). Several lines of evidence have shown the VHL tumor suppressor gene to be the cause of most if not all CCRCCs (20). In addition to the VHL protein alteration, which is a common feature of CCRCCs, other genetic abnormalities are often found in CCRCCs, a number of which being associated with higher grades of tumors (16,21). These late additional genic events are therefore considered as favoring tumor progression (16).

Chromophilic (also called papillary) tumors also originate from the proximal tubule, but they are less aggressive (15). They account for >10% of RCCs (15,16). In this type of tumors, the Embden–Meyerhof pathway is activated but glycogen accumulation is small (19). Eighty percent of them present with an abnormal karyotype, the most frequent one being trisomy 7 and 17. Familial forms are linked to a modification of the MET proto-oncogene, localized to chromosome 7p and coding for the hepatic growth factor receptor (16,22).

Oncocytomas originate from renal collecting duct cells (23). They are benign tumors, rarely inducing metastasis, invasiveness or death (15,16) and are characterized by an increase of mitochondrial density and of mitochondrial DNA (15,24). Oncocytomas might represent a heterogeneous group of tumors with various types of chromosomal abnormalities (16), remarkably producing a similar mitochondrial accumulation together with tumoral growth.

In this work, we have analysed the OXPHOS system in three types of renal tumors, the clear cell type, the chromophilic type and the oncocytomas, in order to address two questions. Does the oxidative capacity depend on the tumor type? Are OXPHOS modifications in the clear-cell group of tumors associated with the clear-cell type itself or with the tumoral grade, i.e. with aggressiveness? If a difference occurred between low- and high-grade CCRCCs, it would probably be linked to genetic events additional to VHL alterations.

Materials and methods

Biopsies

Normal and tumoral tissue samples were dissected from kidneys surgically removed for renal carcinoma. Kidneys were placed on ice and the pathologist examined them before sampling for research. Normal cortex and tumor tissue samples (100–400 mg) were immediately frozen in liquid nitrogen and stored until utilization. Tumor grades were determined according to the Fuhrman's classification (17).

VHL mutations identification

PCR and SSCP analyses were performed using a modified protocol from Gnarra and coworkers (25). The primer pairs #3 and #8, #1 and #10, #9 and #101 were used to amplify exon 1, #102 and #103 for exon 2 and #107 and #6 for exon 3 (25). PCR mixtures (25 µl) contained 20 ng of genomic DNA, 1X PCR buffer, 0.8 µM of each PCR primer, 200 µM of each deoxynucleotide triphosphate and 0.5 U of Taq DNA polymerase (Life Technologies, Cergy-Pontoise, Fr). Reactions involving primers #3/#8, #1/#10 and #102/#103 additionally contained 7.5% dimethyl sulfoxide. An additional set of primers was chosen to screen mutations from nucleotides 84 to 109 because this region is not explored by primer sets #1/#10 nor #9/#101. The sequence for the sense primer named 1–5F was: 5′CGGCGGGGAGGAGTCGGGCG3′ and for the reverse primer named 1–5R, 5′GCGTTGGGTAGGGCTGCGGC3′. The reaction mixtures were incubated in a Gene Amp 9600 thermocycler (Perkin-Elmer Cetus, Courtabouef, Fr) as follows: 94°C for 5 min, 30 cycles of 94°C for 15 s and (a) for primer set #1 and #10, 68°C for 30 s; (b) for primer sets #102 and #103, 62°C for 30 s; (c) for primer sets #107 and #6, 66°C for 30 s; followed by a final extension at 72°C for 5 min. SSCP analysis was performed as previously described (25). Four µl of each PCR product containing 0.5 µCi [^{33}P]dATP were denatured for 10 min at 95°C in 4 µl gel loading buffer and then chilled on ice. Electrophoresis was performed at 10 W for 10 to 16 h at 20°C in a Hydrolink® Mutation Detection Enhanced^{TM} gel (Bioprobe, Montreuil, Fr). Gels were dried and exposed to film from 1–24 h at 20°C. In many cases the fragments with mobility shift were excised from the SSCP gel, eluted in water and reamplified by PCR prior to the sequencing reaction. The PCR products were extracted using the Promega Wizard Prep purification kit. Cycle sequencing was carried out on both strands using either a Sequencing kit (Pharmacia, Uppsala, Sweden) or the Thermosequenase sequencing kit (Amersham, Saclay) on an automated sequencer (Pharmacia, ALF). When direct sequencing revealed intragenic mutation associated with creation or suppression of a restriction endonuclease site, DNA was amplified with the appropriate primers and digested with the informative restriction enzyme according to the manufacturer's guidelines.

Enzyme determinations

Frozen tissue samples of 50–150 mg were minced on a cooled glass plate and then homogenized with a Teflon or a glass Potter in 200–500 µl buffer containing 10 mM Tris–HCl, 0.2 mM dithiotreitol, 0.2 mM EDTA, 0.04 mM pepstatin, 0.4 mM epsilon-amino caproic acid, pH 7.5. Enzyme determinations were performed using the spectrophotometric methods of Srere for citrate synthase (26), and of Rustin *et al.* (27) for complexes II, II + III and IV, respectively. The rate of mitochondrial ATP hydrolysis was measured according to Pullman *et al.* (28) as previously described (29), firstly in the absence of inhibitor and then after addition of 60 mM aurovertin B to the same cuvette. Aurovertin inhibits mitochondrial ATP hydrolysis independently of the state of assembly of complex V (F_0F_1 complex). The complex may be intact or dissociated into F_0 and F_1, or partly depleted of F_0 subunits, and the aurovertin-sensitive ATP hydrolysis activity reflects the function of the F_1 part of the ATP synthase complex.

Mitochondrial DNA contents

Renal tissue (30–40 mg) was homogenized as described above in 80 µl PBS buffer (Life Technologies), then total DNA was extracted with a QIAmp DNA minikit (Qiagen, Courtaboeuf); DNA concentration and purity were determined spectrophotometrically.

Probes for 18S DNA (nuclear) and for 12S DNA (mitochondrial) were prepared by PCR from normal kidney total DNA with the following primer pairs : 5′ATGCGGCGGCGTTATTC3′ and 5′GCGACGGGCGGTGTGTA3′ or 5′CCCCATACCCCGAACCAACC3′ and 5′GGAGTGGGTTTGGGGCT-AGG3′, respectively. The PCR products were purified with a kit (Concert©, Life Technologies).

For Southern blot analysis of 12S DNA/18S DNA ratio, 5 µg total DNA digested by XbaI, according to the manufacturer instructions (Life Technologies, Saclay), were submitted to electrophoresis at 100 V during 6 h on 1% agarose. DNA was then transferred overnight on a nylon membrane (Hybond N+, Amersham) in the presence of 0.4 N NaOH. Probes were labeled with [^{32}P]dCTP using a commercial kit (Radprime, Life Technologies). The membranes were successively hybridized with the 12S and 18S labeled probes (70 ng), according to the membrane manufacturer's instructions. Briefly, after 1 h prehybridization at 65°C without formamide, in the presence of 0.2 mg/ml fish DNA, hybridization of the membranes to the probe was performed overnight, using the same conditions. After several washings, the radioactivity was assessed on a Biorad phosphorimager. The 12S probe revealed two separate bands. The 12S/18S ratio was estimated using the value of the low molecular weight band for the 12S DNA (before 18S probe hybridization) and the value of the single 18S band, which exhibited an intensity similar to that of the small 12S band.

Two-dimensional resolution of the OXPHOS complexes

Crude mitochondria from 10 mg of kidney (wet weight) were solubilized by dodecylmaltoside and the native OXPHOS complexes separated by Blue Native–PAGE using a 5–13% acrylamide gradient gel, as previously described (30). The subunits of the complexes were then resolved in a second dimension by a 16% acrylamide Tricine–SDS gel, and Coomassie-stained (30).

Immunological detection of free F_1-ATPase

Protein spots from 2D gels were transferred to PVDF membranes according to Towbin *et al*. (31). The membrane was incubated firstly with a polyclonal rabbit antibody against bovine ATPase, alpha and beta subunits, diluted 20 000-fold, and secondly with a peroxidase-labeled anti-rabbit antibody. The peroxidase activity was finally revealed with the ECL reagent according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Results

Experimental design and VHL mutations in tumor samples

Tumor samples from a total of 25 patients were used for this work. From histological examination, 16 were classified as

Table I. VHL gene mutations in tumoral tissue

Patient no.	Histological type	Grade	VHL mutation
1	Clear cell	2	None
2	idem	1	263G>A (Trp 88 stop)
3	idem	2	478G>T (Glu 160 stop)
4	idem	2	None
5	idem	1	522deIT (codon 174, stop at codon 201)
17	idem	2	None
17	idem	4	None
6	idem	3	540deIC (codon 180, stop at codon 201)
7	idem	4	440deIT (codon 147, stop at codon 158)
8	idem	4	None
9	Chromophilic		None
10	idem		None
13	Oncocytoma		None
14	idem		None

clear cell RCCs (10 with low grade and six with high grade); five were chromophilic RCCs and four were oncocytomas. VHL gene mutations were analyzed in the DNA extracted from 13 tumors. As shown in Table I, no VHL mutation was found in oncocytomas or in chromophilic tumors, whereas five mutations were identified out of nine clear cell RCC. No VHL mutation was found in the normal kidney counterpart of any tumor sample.

Table I shows that four mutations in the beginning of exon 3 should modify the protein or eliminate its C-terminal alpha domain necessary for elongin C binding (32), if the protein is expressed. Another mutation (patient 2) not only induced the loss of the alpha domain but also that of most of the hydrophobic beta sheet domain (32), necessary for nuclear-cytoplasmic traffic (33).

Oxidative phosphorylations and mitochondrial DNA content in different types of renal tumors

The oxidative capacity of tissue specimens from 25 patients was estimated by measuring four OXPHOS activities localized to the inner membrane. Complex II, or succinate dehydrogenase, is both participating in the respiratory chain and in the Krebs cycle; it was estimated from the malonate-sensitive succinate dichlorophenol indophenol oxidoreduction rate. Succinate cytochrome *c* oxidoreductase (complexes II + III), was determined as the malonate-sensitive activity. Almost no malonate-insensitive activity could be detected and the sensitivity to antimycin was not significantly different from that to malonate. Cytochrome oxidase (complex IV) was determined by the cyanide sensitive oxidation of reduced cytochrome *c*, and ATP hydrolysis (assigned complex V in Figures 1 and 2) was determined as the aurovertin-sensitive activity, which reflects the function of the F_1 part of the ATP synthase complex.

These OXPHOS activities were compared with the mtDNA content (expressed as 12S/18S ratio) and citrate synthase (CS), a mitochondrial matrix enzyme participating in the Krebs cycle, which is often used as marker for the mitochondria content of a tissue. Citrate synthase and all of the complex II protein subunits are encoded by the nuclear genome, whereas the other complexes contain some subunits encoded by the mitochondrial DNA and the remaining by the nuclear genome.

Enzyme activities in the healthy counterpart of tumors were in the normal range, with large individual variations. They were comparable to those reported by Chrétien *et al. (*34). However, in oncocytomas, the enzyme activity of non-tumor tissue was frequently above the normal range (not shown).

Figures 1, 2 and 3 clearly show that, in all renal tumors, mitochondrial enzymes and DNA are generally modified as compared with those of normal. Moreover, there are significant differences between tumor types. Indeed, the results summarized in Figure 3 and the non-parametric statistical analysis reported in Table II show a clear-cut difference between the benign oncocytomas and the clear-cell or the chromophilic carcinomas. In oncocytomas (Figure 2, lower panel) all mitochondrial markers were increased but to a variable degree. Complex IV, citrate synthase, and mtDNA were on the average increased by factors of about 7, 5 and 4, respectively, while complexes V, III, and II were only slightly increased or close to normal. On the contrary, in other tumor types such as chromophilic and low-grade CCRCC tumors, all parameters were decreased but complex V. In the most aggressive high-grade CCRCCs, complex V activity was also significantly decreased.

Another important result is the gradual worsening of the mitochondrial impairment from the less aggressive chromophilic carcinomas to the high-grade CCRCCs (Figure 3 and Table II). In chromophilic tumors (Figure 2, upper panel) the OXPHOS activities were less reduced than in CCRCCs of all grades (Figure 1). The aurovertin-sensitive ATPase activity was by a factor of two higher than expected from the activities of complexes II, III, and IV. In this respect chromophilic tumors showed similarity to some low grade CCRCCs. The differences were clearly associated with the tumor type or grade rather than to their stage of progression (Table II).

We compared the changes of OXPHOS enzymes with those of citrate synthase and of mtDNA, which reflect the mitochondrial content of a cell. Selvanayagam and Rajaraman (35) had already observed mtDNA depletion in CCRCCs. In this study, changes in citrate synthase activity were grossly correlated to that of mt DNA variations (Figures 1, 2 and 3). The average decrease of citrate synthase and of mt DNA content was not as important in high-grade CCRCCs as in low-grade (Figure 1). This original finding is in agreement with previous histological observations showing that mitochondrial content is not always decreased in high-grade CCRCCs, especially in eosinophilic zones, probably resulting from additional gene alterations (17). In addition, it cannot be excluded that the 12S/18S decrease observed in some high grade CCRCCs might be due to the polyploidy sometimes put forward in high-grade CCRCCs rather than to mtDNA depletion. Nevertheless, in spite of the observed variability of the mitochondrial content and of the well-known variability of genetic events in high-grade CCRCCs, OXPHOS activities were consistently and dramatically decreased in these tumors. Therefore, we conclude that aggressiveness of renal tumors is correlated with the OXPHOS impairment rather than with the decreased mitochondrial content.

In some patients, samples were obtained from different zones of the tumor, but no obvious differences in enzyme activities could be put forward inside these individual tumors. In patient 7, the red zone was made of typical clear cells with hemorrhage, whereas in the white zone, tumor cells were

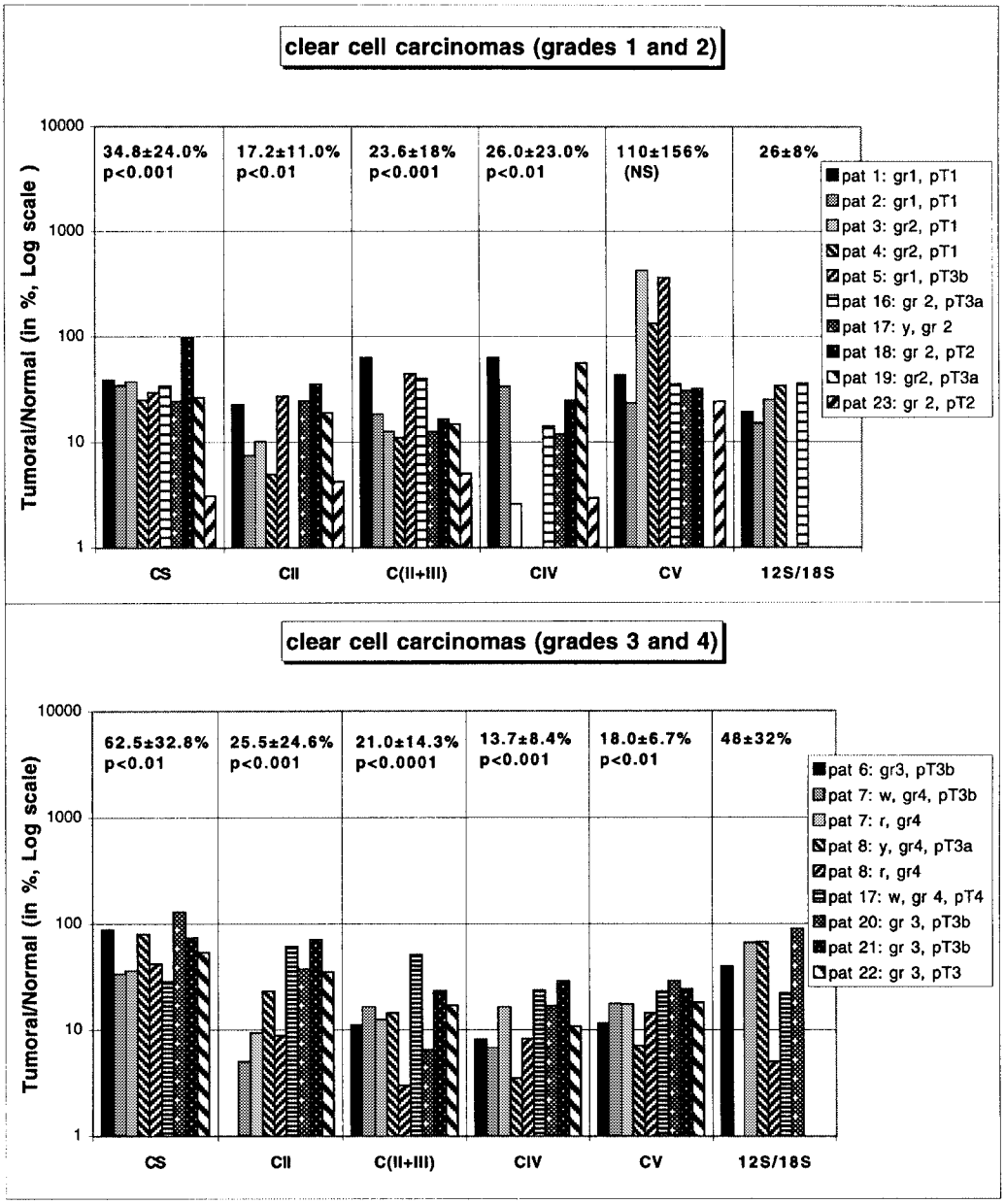

Figure 1. OXPHOS activities and mitochondrial DNA contents in clear cell carcinomas. Kidneys were excised from patients suffering from renal cell carcinomas. The grade (gr) and stage (pT) are indicated for each tumor sample. Normal cortex samples and tumoral samples were processed as described in Materials and methods and the following parameters were determined: enzyme activity of citrate synthase (CS), succinate DCPIP reductase (CII), succinate cytochrome *c* reductase (CII+III), cytochrome oxidase (CIV), aunovertin-sensitive F1-ATPase (CV), and ratio of mitochondrial to nuclear DNA (12S/18S). In patients 7, 8 and 17, samples were dissected from two different regions of the tumor, white (w) and red (r) tissue for patient 7, yellow (y) and red for patient 8, yellow and white for patient 17. The results are presented for each patient as the tumoral activity (or 12S/18S ratio), expressed in percent of the normal tissue value from the same kidney. The mean percent value ± SD is given above each group of patients, with the *P* error value indicating the significance of the differences between tumor and normal tissues (Student's test for paired data).

eosinophilic. In patient 8, yellow and red zones were made of clear cells, with more blood in the red zone. No difference appeared within these high-grade tumors. In patient 17, a sarcomatoid, grade 4 clear-cell tumor, contained a small, hemorrhagic, low-grade tumor zone; however, even in this case, mitochondrial activities were similar in the two tumor zones of different grades, except for complex V activity, which was slightly more decreased in high-grade zone (23% remaining instead of 30%).

Reduced assembly/stability of ATP synthase complex in renal carcinomas

Aurovertin-sensitive mitochondrial ATPase activity was not consistently decreased in renal carcinomas, nor was it uniformly increased in oncocytomas, in contrast to the other OXPHOS complexes (Figures 1, 2 and 3). This suggested that ATPase/ATPsynthase regulation and regulation of other OXPHOS complexes are independent. Whereas aurovertin-sensitive ATPase activity was always decreased in high-grade CCRCCs,

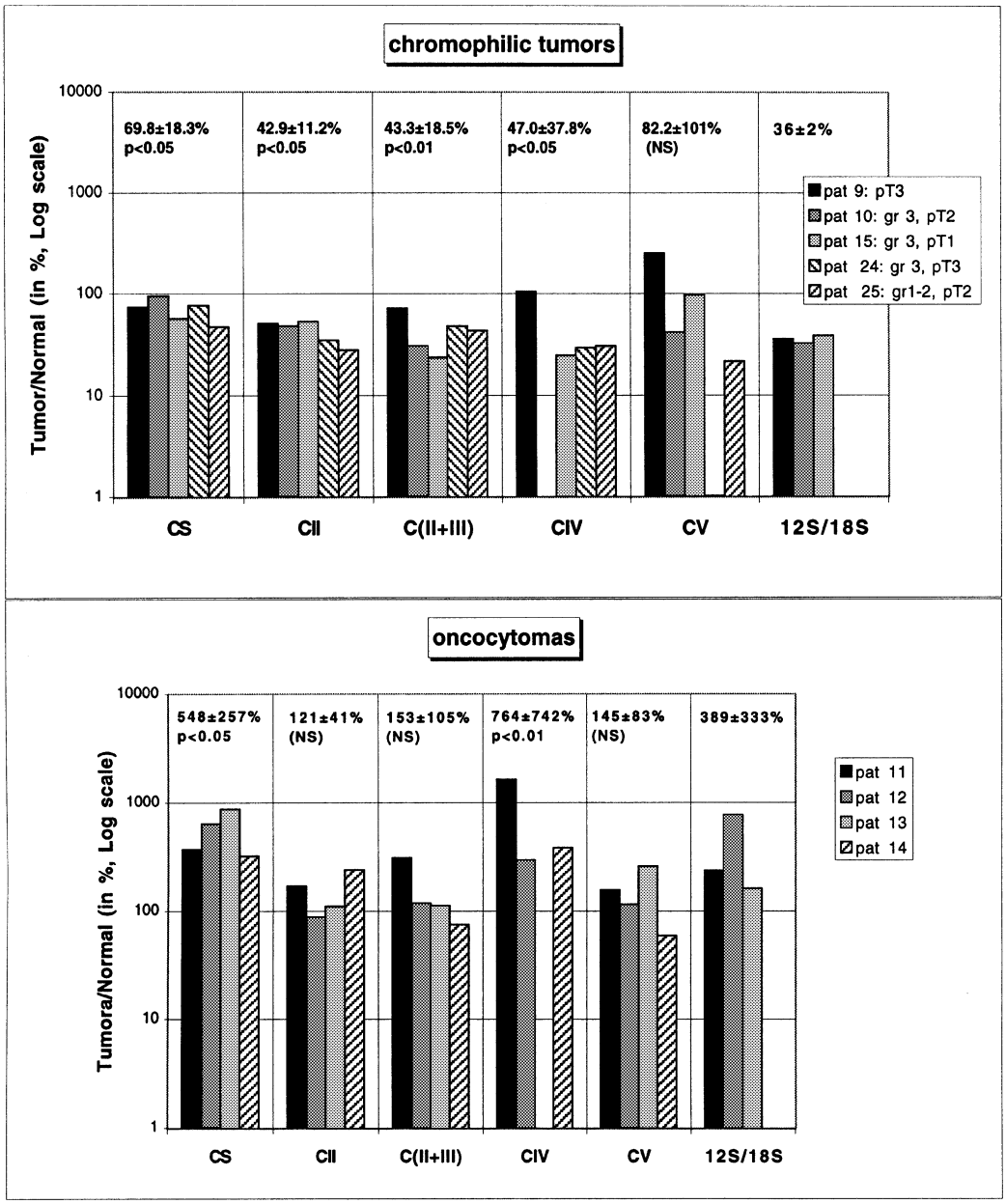

Figure 2. OXPHOS activities and mitochondrial DNA contents in other renal cell tumors. Legend see Figure 1.

it was sometimes increased in low-grade CCRCCs and in chromophilic tumors (Figures 1 and 2). It should be mentioned that sample from patient 19 (low-grade CCRCC) which exhibited no aurovertin-sensitive ATPase activity presented in the absence of inhibitor with an ATPase activity representing about ~30% of the normal rate. It was difficult to tell whether the aurovertin sensitivity had been lost in F_1 or whether the F_1 content had been strongly decreased in this sample. The same possible explanations also hold for other tumor samples exhibiting decreased aurovertin-sensitive ATPase activity.

In order to better understand why aurovertin-sensitive ATP hydrolysis activity was variable, we determined the complex V protein amounts in 20 patients, using blue-native PAGE (BN–PAGE) for resolution of the native OXPHOS complexes from 10 mg normal and tumor tissue, followed by resolution of the protein subunits by a second dimension SDS–PAGE, and Coomassie-staining (36,37). Typical results from each tumor type are shown in Figure 4. The most intensely stained alpha and beta subunits of ATP synthase (complex V) were detectable in most tumor samples, even from CCRCCs and chromophilic RCCs with considerably reduced oxidative capacity, and were used to quantify the complex V protein amounts (Figure 4 and Table III). Complexes I to IV in tumors often were too low for detection, except in oncocytomas with increased amounts of OXPHOS proteins.

Unlike ATPase activities, complex V protein amounts were uniformly decreased in clear cell and chromophilic type carcinomas and increased in benign oncocytomas (Table III), and therefore were comparable to the reduced or increased catalytic activities of other OXPHOS complexes (Figures 1 and 2). This discrepancy between high ATP hydrolysis activity and low complex V protein amount suggested an instability

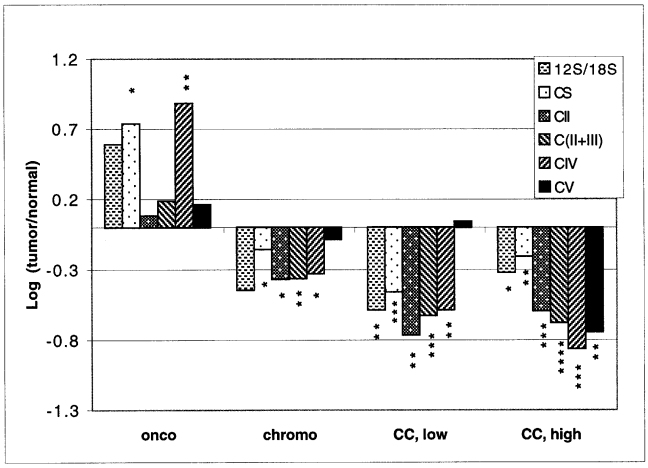

Figure 3. Relationship between OXPHOS activities and tumor types. Mean values for each parameter taken from Figures 1 and 2 are presented here in log of the ratio: tumor value/normal value. For each parameter in each tumor type, normal and tumor individual values were compared using the Student's test for paired data. *$P<0.05$; **$P<0.01$; ***$P<0.001$; **** $P<0.0001$.

Table II. Statistical analysis of OXPHOS activities in renal tumors

	CS	CII	C(II+III)	CIV	CV	12S/18S
A-Tumor type						
clear cell low-gr/high-gr	*	NS	NS	NS	***	NS
clear cell low-gr/chromophilic	*	**	NS	NS	NS	NS
clear cell high-gr/chromophilic	NS	NS	*	**	NS	NS
oncocytoma/chromophilic	*	*	*	*	NS	*
oncocytoma/clear cell, low gr	**	**	**	*	NS	*
oncocytoma/clear cell, high gr	**	**	**	*	*	*
B-Tumor stage						
clear cell, stages 1/2/3/4	NS	NS	NS	NS	NS	NS
clear cell and chromophilic, stages 1/2/3/4	NS	NS	NS	NS	NS	NS

The data from figures 1 and 2 analyzed with the Kruskall and Wallis' test were significantly different when they were ordered according to the tumor type, but not according to the tumor stage (B). Differences between tumor types were then analyzed with the Mann and Whitney's test for unpaired data (A). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant.

of F_1F_0 ATP synthase (complex V). Indeed, dissociation of F_1-sector from F_0 leads to loss of ATP synthase activity, but the ATPase activity can increase up to a factor of 10 (38).

Disturbed assembly/stability of complex V was assayed by immunodetection of the alpha and beta subunits of complex V in electroblotted 2D gels. No or very small amounts of free F_1 were detected in all non-tumor tissues, and in all oncocytomas, as exemplified in Figure 5, A–D. However, free F_1-part of ATP synthase was often but not generally observed in western blots of CCRCC and chromophilic tumors, and the ratio of free F_1/holo-complex V varied considerably. For example, in CCRCC patients 20 and 21 and in patient 15 with a chromophilic tumor (Figure 5, I, K, and L) the amounts of free F_1 were higher or comparable to the amounts of holo-complex V, while in CCRCC patients 1 and 23 (Figure 5, E and F) with extremely low amounts of holo-complex V the detection limit for free F_1 was not reached.

These results clearly showed that complex V assembly/stability is reduced in at least several CCRCC and chromophilic tumors. However, the increased amounts of free F_1-sector and the F_1/complex V ratio did not correlate well with the increased

Table III. Complex V contents in tumors

Patient no.	Histological type (Grade)	Complex V in tumors protein amount from 2D gels (% of normal)
1	clear cell (1)	10
4	clear cell (2)	8
5	clear cell (1)	67
16	clear cell (2)	<5
17 (yellow tumor)	clear cell (2)	5
18	clear cell (2)	14
19	clear cell (2)	25
23	clear cell (2)	10
mean ± SD		20 ± 22**
6	clear cell (3)	41
7 (red)	clear cell (4)	19
8 (red)	clear cell (4)	19
17 (white)	clear cell (4)	5
20	clear cell (3)	26
21	clear cell (3)	49
mean ± SD		27 ± 16**
9	chromophilic	40
10	chromophilic	36
15	chromophilic	26
mean ± SD		34 ± 7
11	oncocytoma	225
12	oncocytoma	207
13	oncocytoma	317
14	oncocytoma	173
mean ± SD		231 ± 62*

The results in tumor tissues were compared to those of their normal counterparts by the Student's test for paired data. *$P<0.05$; **$P<0.01$.

aurovertin-sensitive ATPase activities in CCRCCs. Free F_1 might explain some of the results observed in chromophilic tumors. Indeed tumor from patient 9 has increased ATPase activity (Figure 2), reduced amount of intact complex V protein (Table III) and high amounts of free F_1 (Figure 5). Similarly, tumor from patient 15 had normal ATPase activity (Figure 2) in spite of a reduced amount of complex V (Table III), but free F_1 (Figure 5) could compensate for the lack of complex V and explain the maintained ATPase activity. By contrast, in most CCRCCs, free F_1 was associated with a low aurovertin-sensitive ATPase activity (Figures 1 and 5) as well in low-grade tumors (G and H, from patients 19 and 18) as in high-grade tumors (I and K from patients 20 and 21).

We conclude that the lability of F_0F_1, is clearly demonstrated in all types of RCCs. It is associated in rare cases with a gain of aurovertin-sensitive ATPase activity, and in most cases with a loss of this activity.

Discussion

The set of mutations found in this work is in agreement with previous data on VHL mutations in RCCs (16,20,39) which report the presence of VHL mutations in >50% of CCRCCs. The absence of detectable mutation in the other CCRCCs does not imply that normal VHL protein is expressed. Indeed, a loss of heterozygosity in the region of VHL, reflecting a large deletion on one allele, is present in 98% of CCRCCs, and the other VHL allele is either modified by mutation in >50% of the cases (16,20,39) or inactivated by hypermethylation in 20% of the cases (20). The remaining few percent of CCRCCs originate from not clearly identified events, possibly reducing pVHL expression, or due to still unidentified tumor suppressor genes also included in the frequently lost region of VHL.

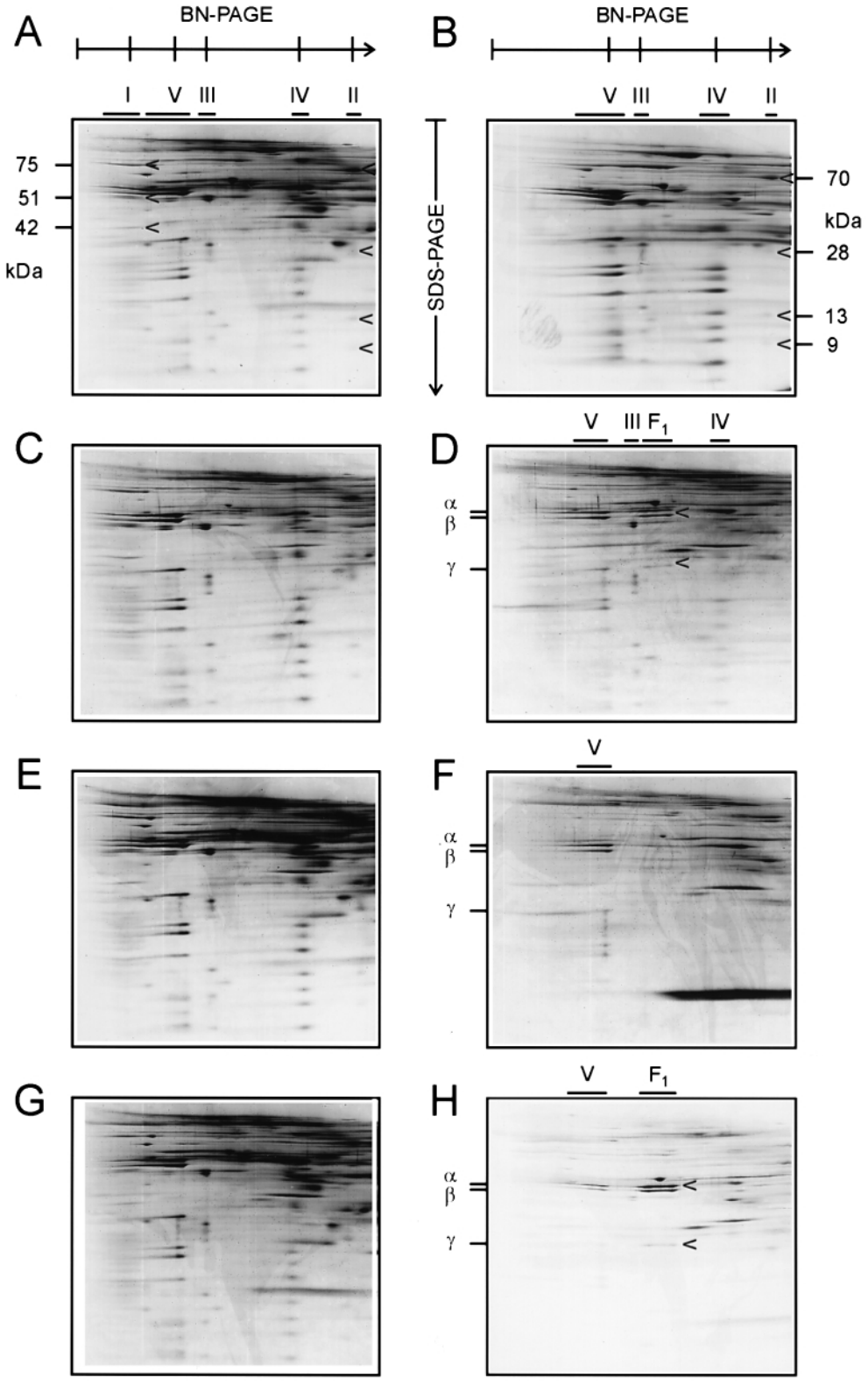

Figure 4. Two-dimensional resolution of the OXPHOS complexes from tumor tissues and matched control kidney cortices. Crude mitochondria obtained from 10 mg of normal kidney or tumor tissue (wet weight) were solubilized by dodecylmaltoside and the native OXPHOS complexes separated by BN-PAGE using a 5–13% acrylamide gradient gel. The subunits of the complexes were resolved in the second dimension by a 16% acrylamide Tricine-SDS-gel, and stained with Coomassie blue. A and B: oncocytoma (patient 13), normal (A), and tumor (B). C and D: chromophilic tumor (patient 15), normal (C) and tumor (D). E and F: CCRCC, low grade (patient 19), normal (E) and tumor (F). G and H: CCRCC, high grade (patient 20), normal (G) and tumor (H). In A and B, three bands of complex 1 (75 kDa, 51 kDa, and 42 kDa) and four bands of complex II (70 kDa, 28 kDa, 13 kDa, and 9 kDa), which are just detectable after Coomassie-staining are marked by arrows. In D and H, the arrows indicate the complex V, alpha, beta and gamma subunit bands.

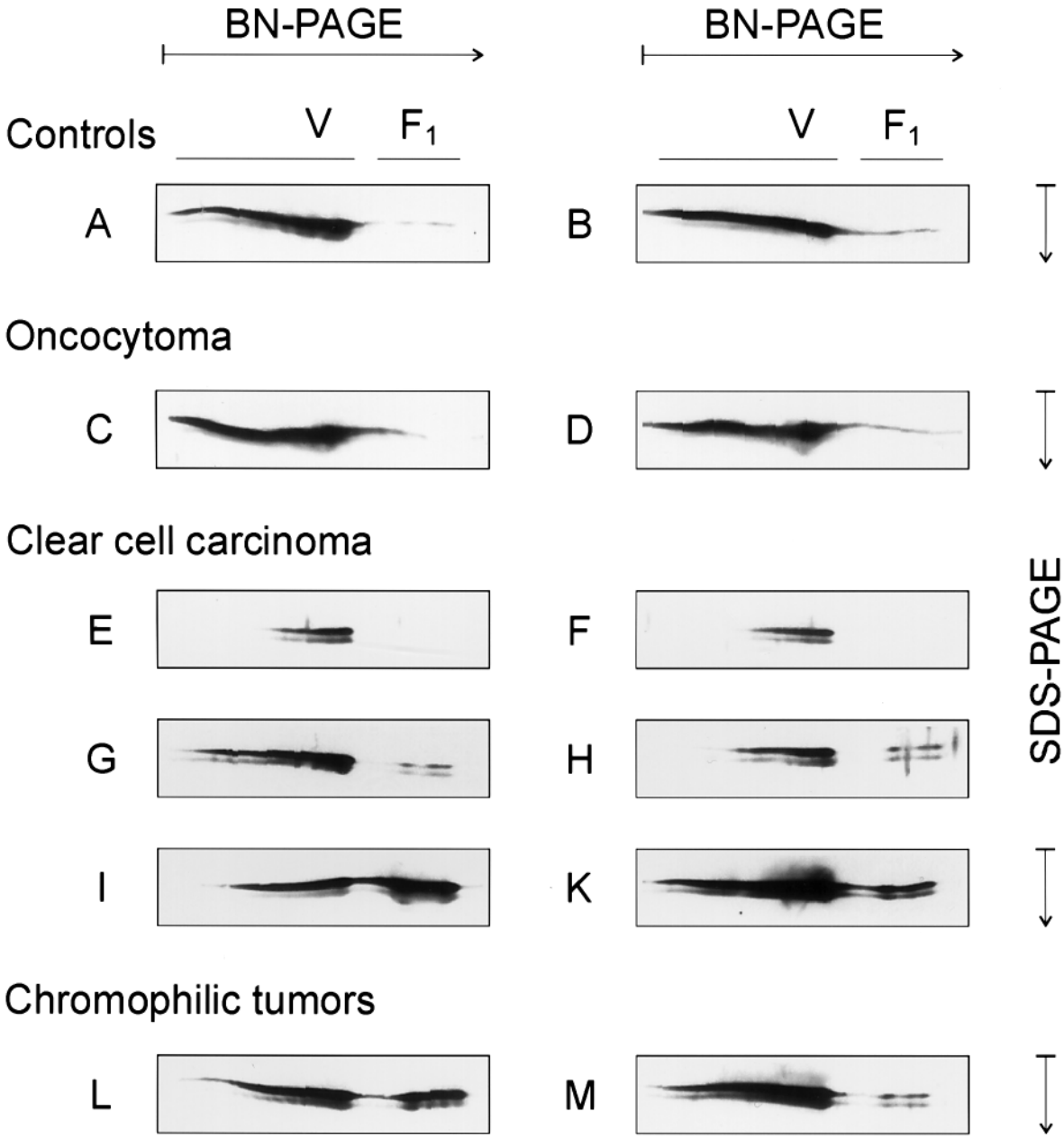

Figure 5. F0F1 content and instability in tumor tissues. Kidney specimens were processed for BN-PAGE and second dimension SDS-PAGE as described in Figure 4, and the western blots issued from the gels were stained using an antibody against subunits alpha and beta. Only the areas including the 40–60 kDa subunits of F0F1 ATP synthase (M_r ~700 kDa; represented by the alpha and beta subunits), and free F1 ATPase (~400 kDa; represented by the alpha and beta subunits) were selected. A and B: normal kidney (patients 13 and 17). From C to M, tumor tissues: C and D from patients 12 and 11 (oncocytomas), E to H, from patients 1, 23, 19, and 18 (low-grade CCRCCs), I and K from patients 20 and 21 (high-grade CCRCCs), L and M from patients 15 and 9 (chromophilic), respectively.

This work is the first report describing mitochondrial enzyme activities in various RCCs. The first conclusion of the study is that a clear-cut difference exists between the mitochondrial properties of the three types of tumors examined. Therefore, the genic events characterizing a tumor type induce a somewhat specific metabolic profile, at least in the domain of oxidative metabolism. The present results can be useful for differential diagnosis of RCC.

Interestingly, a study of mRNA differential expression in RCCs has recently been published, showing that mitochondrial transcripts are amongst the main markers differentiating tumor type (40). Although the latter study only compared different types of tumors and not, as in our present study, tumors and their normal counterpart, it shows that oncocytoma, when compared with CCRCC, overexpressed many transcripts related to OXPHOS. For example, genes encoding some mitochondrial complex III or complex IV subunits were overexpressed as well as those of a few ATPsynthase subunits.

Oncocytomas have been known for a long-time to present with elevated mitochondrial DNA and cytochrome oxidase (23,24). Our study confirms these data. Moreover, analyzing enzyme activities in tumor and normal tissues, and determining protein amounts by 2D electrophoresis, we obtained a complete and detailed view of the whole OXPHOS system in oncocytomas. An ~5-fold increase of citrate synthase indicated actively proliferating mitochondria, and an increase of the number of

mitochondria. This increase is paralleled by an ~4-fold increase of the 12S/18S ratio, indicating elevated amounts of mtDNA, and an ~7-fold increase of cytochrome *c* oxidase activity (complex IV). However, the activities involving succinate dehydrogenase (complex II), quinol cytochrome *c* reductase (complex III), as well as ATPase (complex V) were only slightly increased, and complex I which was analysed only by 2D electrophoresis (cf. Figure 4B) was not detectable. This suggested that mitochondria might proliferate in oncocytoma to compensate for an overall decreased OXPHOS capacity with complexes I, II, III, and V, but not complex IV, as potentially limiting enzymes. The elevated mitochondrial biogenesis might be similar to the often observed up-regulation in muscle of patients suffering from mitochondrial myopathies, and the development of ragged red fibers (41). A 2-fold decreased rate of ATP synthesis has recently been documented in thyroid oncocytoma by Savagner *et al.* (42) although the mitochondria proliferated, and a 3- to 4-fold increase of mtDNA and of some mitochondrial transcripts was observed.

An important result of our study is that the impairment of the OXPHOS enzyme activities is grossly correlated with aggressiveness of the different tumor types (graduation from oncocytomas, chromophilic tumors, low-grade, then to high-grade carcinomas). Aggressiveness of a tumor type involves proliferation rate, invasiveness, as well as long-term survival in the epidemiologic data. Since the mechanism of tumorigenesis could concern mitosis, apoptosis, loss of aggregation or loss of contact inhibition, it would have been of interest to know the mitotic index in routine tumor histology. In normal kidney, the mitotic index is about zero, and in CCRCCs, this index is increased (17). Though individual data of mitotic index are not available in this study, our findings are in agreement with the view that a competition may exist between substrate oxidation and substrate utilization in the biosynthetic processes necessary for tumorigenic mitosis.

We speculate that the different aggressiveness of CCRCC compared with chromophilic RCC is related to the different metabolic functions of the two genes frequently linked to these tumors, *VHL* gene and *MET* gene, respectively, since CCRCC and chomophilic RCCs originate from the same cell type. Therefore the dual, coordinated role of VHL protein in the regulation of substrate pathways and in the cell cycle of oxidative tissues (3,11,43) would be the cause of tumor aggressiveness in these tissues. However the changes in energy metabolism are only a part of the numerous modifications induced by a tumor suppressor alteration. Each of these modifications could be involved and necessary for tumoral growth and invasiveness. Another type of metabolic change associated to a tumor suppressor gene has been described in a different tumor cell type, the hepatoma: the mitochondrial porin-bound hexokinase II is increased under the control of mutated p53 (13). This would direct mitochondrial ATP preferentially to glucose-6-phosphate synthesis and, hence, is expected to increase the biosynthetic pentose phosphate pathway.

However, the progression of aggressiveness is not exclusively associated to one tumor suppressor gene or to one oncogene, since there is a significant increase of aggressiveness from low- to high-grade CCRCCs, probably due to variable additional genetic events, and associated with increased ATPase defect. This suggests that any event leading to the impairment of ATPase enzyme activity might favor tumor aggressiveness.

The lability of complex V, often observed in CCRCCs and in chromophilic tumors, seems to indicate that the mitochondria were in a bad structural and functional state. However, it cannot be excluded at present that, in some cases, the structural alteration of complex V offers a functional advantage in cells exhibiting a deficient respiratory chain. In rho° cells, depleted of mitochondrial DNA, the OXPHOS complexes containing mitochondria-encoded subunits disappear. However, the F_1-ATPase remains stable and active in spite of the absence of mitochondria-encoded F_0 subunits. The F_1-ATPase activity is necessary to maintain the mitochondrial membrane potential (29,44), thus providing a strong argument favoring the hypothesis that ATPase is regulated independently from the other OXPHOS complexes. In rho° cells, this property allows mitochondria to use the cytoplasmic ATP originating from glycolysis, and, by means of electrogenic adenine nucleotide translocation, to maintain the mitochondrial membrane potential, required for mitochondrial matrix activities (29,44). It is therefore relevant that such a metabolic adaptation could also exist after alteration of oxidative metabolism in low-grade CCRCC and in chromophilic tumors. Since it is well established that *VHL* gene expression is altered in most CCRCCs (20), this suggests that the adaptive increase of F_1-ATPase activity is very likely an early event and could be lost when additional genetic events occur in high-grade and some low-grade CCRCCs.

The weak correlation between aurovertin-sensitive ATP hydrolase activity and free F_1 protein content remains however to be explained. It strongly suggests that, in tumors, additional factors are sometimes altering F_1-ATPase activity. A possible hypothesis could be a loss of F_1 affinity for aurovertin. Another possible hypothesis could be variations in the expression or activity of the F_1 inhibitor protein (IF_1), which is able to inhibit the ATPase component of ATPase/ATPsynthase (45,46). The putative variations of this molecule in renal tumors will be the subject of future investigations in our laboratory.

In conclusion, clear-cut differences of mitochondrial capacities occur between three types of renal tumors. In CCRCCs, *VHL* inactivation is linked to a severe decrease of oxidative phosphorylations. The only exception concerns the F_1-ATPase activity, which is not always decreased. Its inactivation is possibly related to an additional event. The relationship between mitochondrial activity decrease and tumor aggressiveness is in agreement with the hypothesis that mitochondrial capacity decrease favors a faster growth or an increased invasiveness.

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