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Loss of mitochondria in ganglioneuromas

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1. ABSTRACT

A shift in cellular energy production from oxidative phosphorylation (OXPHOS) to glycolysis, even under aerobic conditions, is called the Warburg effect. To elucidate changes of the mitochondrial energy metabolism in ganglioneuroma (GN) individual OXPHOS enzymes were analyzed by activity assays and by immunohistochemical staining methods. GN (n=7) showed a significant reduction in the activity and content of OXPHOS enzymes. Citrate synthase activity was also severely diminished in GN compared to normal cortical kidney (p=0.0002) and adrenal (p=0.0024) tissues. Furthermore, the mitochondrial membrane protein porin was undetectable or significantly reduced. Accordingly, a reduction of the copy number of mitochondrial DNA was observed in GN compared to cortical kidney tissue. The striking decline of mitochondrial mass is specific for GN but not for neuroblastoma, in which a reduction of the OXPHOS complexes without reduction of mitochondrial mass was reported. Knowledge of the mechanism by which tumor cells achieve the Warburg effect will provide a starting point for functional studies aimed at restoring aerobic energy metabolism as a potential new therapeutic strategy to treat malignancies.

2. INTRODUCTION

Ganglioneuromas (GNs) are invariably benign neoplasms composed of mature ganglionic neurons scattered singly or in clumps within a relatively abundant and dense stroma of neurofibrils and collagenous fibers (1). GNs, ganglioneuroblastomas, neuroblastomas (NBs), paragangliomas and pheochromocytomas all originate from the neural crest. According to the Shimada classification, GNs are Schwannian stroma-dominant and NBs are Schwannian stroma-poor neuroblastic tumors (2, 3). Because a substantial proportion of histologically proven GNs exhibit metabolic activity such as increased secretion of catecholamines and metaiodobenzylguanidine uptake similar as NBs, are diagnosed at a higher age, and the high percentage of immature GNs indicates that most of the GNs develop through differentiating NBs (4). Maturation of NBs starts from a morphologically undifferentiated or poorly differentiated neuroblastic tumor, passes through transitional states, and ends with the formation of a mature GN (1-3). Despite slow growth, they may attain large dimensions and become clinically evident because of compression of neighboring structures (4).

More than 80 years ago Otto Warburg suggested that cancer is associated with a decrease in mitochondrial energy metabolism paralleled by an increase in glycolytic flux (5). Mitochondrial defects have been proposed to play an important role in the initiation and progression of different types of cancer (6, 7). It has been demonstrated that activation of oncogenes or inactivation of tumor suppressor genes, such as p53 and the VHL (von Hippel-Lindau), can lead to an up-regulation of glycolytic enzymes or a down-regulation of OXPHOS complexes (8-11). Accordingly, coordinated down-regulation of all OXPHOS enzymes has been observed in several types of tumors, including renal cell carcinomas and NBs (12, 13).

Moreover, tumors with specific defects affecting single components of the OXPHOS system have been described. One example is the loss of both assembly and function of complex I in oncocytic tumors, frequently caused by severe pathogenic mutations in mitochondrial DNA-encoded respiratory chain complex I subunits (14-16). Paragangliomas and pheochromocytomas are associated with succinate dehydrogenase (SDH, complex II) mutations (17). Very recently another mechanism that induces a glycolytic phenotype, leading to down-regulation of OXPHOS enzymes, was identified. Mutations in the isocitrate dehydrogenase genes, *IDH1* and *IDH2* were found in glioma multiforme (18, 19). The R132 mutation of IDH1 generates a new enzyme with α-ketoglutarate reductase activity that produces 2-hydroxyglutarate, which strongly correlates with cancer formation. This effect may be due to the fact that 2-hydroxyglutarate inhibits prolyl 4-hydroxylase (PHD) activity by competing with α-ketoglutarate binding (19). Inhibition of PHD in turn leads to stabilization of hypoxia-inducible factor (HIF), which is a key event for the induction of the glycolytic switch in tumor cells.

We hypothesize that GNs, as other tumors originating from neural crest cells, have impaired aerobic mitochondrial energy metabolism. Therefore, in the present study we investigated alterations of OXPHOS complexes and mitochondrial mass in human GN tissues.

3. MATERIAL AND METHODS

3.1. Ethics Statement

The study was performed according to the Austrian Gene Technology Act. Experiments were performed in accordance with the Helsinki declaration of 1975 (revised 1983) and the guidelines of the Salzburg State Ethics Research Committee being no clinical drug trial or epidemiological investigation. All patients have 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. Samples

GNs from 7 patients (5 female, 2 male) were obtained from the Department of Pediatrics, Salzburg, Austria. As references for enzymatic measurements, three age-matched adrenal glands were kindly provided by the tumor tissue bank of the University Hospital, Cologne, and one adrenal gland was obtained from the Department of Pathology, Salzburg. Sample information on NBs and control kidneys was previously reported (12).

The mean age of the GN patients at surgery was 78 months. According to the Evans staging system all tumors were stage 1 (20). One patient had received chemotherapy before surgery. Tissues were frozen and stored in liquid nitrogen within 20 minutes after surgery. Tumor cell content and cellular composition of the samples were evaluated using hematoxylin- and eosin-stained frozen sections. Tissue samples with a tumor cell content of more than 90% were taken for enzymatic measurements and Western blot analysis. Fifteen unaffected kidney tissues and 4 adrenal tissues served as references for the enzymatic measurements. Data of GNs were statistically analyzed in comparison to NBs, and kidney tissues as reported previously (12).

3.3. Spectrophotometric detection of OXPHOS enzyme activities and citrate synthase

GN and adrenal gland tissues (20-100 mg) were homogenized with a tissue disintegrator (Ultraturrax, IKA, Staufen, Germany) in extraction buffer (20 mM Tris-HCl, pH 7.6, 250 mM sucrose, 40 mM KCl, 2 mM EGTA) and finally homogenized with a motor-driven Teflon-glass homogenizer (Potter S, Braun, Melsungen, Germany). The homogenate was centrifuged at 600*g* for 10 min at 4°C. The postnuclear supernatant (600*g* homogenate) containing the mitochondrial fraction was used for measurement of enzyme activities and Western blot analysis. Citrate synthase was determined according to Srere (21) with modifications. Briefly, the reaction mixture contained 50 mM Tris- HCl pH 8.1, 0.1% bovine serum albumin (BSA), 0.1% TritonX-100, 0.2 mM 5,5'-dithio-bis(2-nitrobenzoic acid), 0.15 mM acetyl-CoA and the 600g homogenate. After initially recording thiolase activity for 2 min the citrate synthase reaction was started by addition of 0.5 mM oxaloacetate and was followed at 412 nm for 8 min. The mean unspecific thiolase activity in NBs was 2% of the citrate synthase activity.

Enzyme activities of the OXPHOS complexes were determined as previously described (13, 22). Briefly, rotenone-sensitive complex I activity was measured spectrophotometrically as NADH/decylubiquinone oxidoreductase at 340 nm. The enzyme activities of citrate synthase and complex IV (ferrocytochrome c/oxygen oxidoreductase), and the oligomycin-sensitive ATPase activity of the F_1F_0 ATP synthase were determined by using buffer conditions as previously described by Rustin *et al.* (1994) (23). The whole reaction mixture for the ATPase activity measurement was treated for 10 seconds with an ultra-sonifier (Bio cell disruptor 250, Branson, Vienna, Austria). Complex II activity was measured according to Rustin *et al.* with the following modifications. The reaction mixture contained 50 mM potassium phosphate pH 7.8, 2 mM EDTA, 0.1% BSA, 3 µM rotenone, 80 µM 2,6-dichlorophenol, 50 µM decylubiquinone, 1 µM antimycin A, 0.2 mM ATP, 0.3 mM KCN and the 600g homogenate. The mixture was preincubated for 10 min at 37°C, started

by addition of 10 mM succinate, and followed for 6 min at 600 nm.

The reaction mixture for the measurement of the complex III activity contained 50 mM potassium phosphate buffer pH 7.8, 2 mM EDTA pH 8.6, 0.3 mM KCN, 100 µM cytochrome c, 200 µM reduced decyl-ubiquinol. The reaction was started by addition of the 600g homogenate. After 3 – 4 min the reaction was inhibited by addition of 1 µM antimycin A. Antimycin A- insensitive activity was substracted from total activity to calculate complex III activity. All spectrophotometric measurements (Uvicon 922, Kontron, Milan, Italy) were performed at 37°C.

3.4. Western blot analysis

A total of 5 µg protein of the 600*g* homogenate was separated on 10% acrylamide/bisacrylamide gels and transferred to nitrocellulose membranes using CAPS buffer (10 mM 3-[cyclohexylamino]-1-propane sulfonic acid, pH 11; 10% methanol). The membranes were washed in Tris-buffered saline (TBS) for 5 min, air-dried for 30 min, washed 10 min in TBS and blocked 1 h at room temperature in 2% blocking powder (Roche, Mannheim, Germany) dissolved in TBS. After washing, the membranes were incubated with the primary antibody diluted in 2% blocking powder dissolved in TBS-Tween 20. The following primary antibody dilutions and incubation times were used: monoclonal mouse anti-SDHA 70 kD antibody (1:15,000, 2 h, room temperature (RT); MitoSciences, Eugene, Oregon, USA], monoclonal mouse anti-Core 2 antibody (1:2000, 2 h, RT; MitoSciences), monoclonal mouse anti-porin (1:2000, 2 h, RT; MitoSciences), polyclonal rabbit anti-GAPDH antibody (1:5000, 1 h, RT; Trevigen). After washing, the membranes were incubated with secondary antibodies as follows: SDHA 70 kD, Core 2 and porin, 1 h with labeled polymer-HRP-antimouse 1:1000 (EnVision kit, Dako) at RT; GAPDH, 1 h with labeled polymer-HRP-anti-rabbit 1:1000 (EnVision kit, Dako) at RT. Detection was carried out with Lumi-Light^{PLUS}POD substrate (Roche). After detection of SDHA 70 kD Fp, complex III subunit Core 2, and porin, the nitrocellulose membranes were washed twice in stripping buffer (25 mM glycine-HCl, pH 2, 2% SDS) for 15 min, and a subsequent immunodetection with anti-GAPDH antibody was performed as described above.

3.5. Immunohistochemical staining and analysis

For immunohistochemical staining, the following antibodies were used: mouse monoclonal anti-complex I subunit NDUFS4 (1:1000; Abcam, Cambridge, UK), mouse monoclonal anti-complex II subunit 70 kDa Fp (1:5000; MitoSciences), mouse monoclonal anti-complex III subunit Core 2 (1:1500; MitoSciences), mouse monoclonal anti-complex IV subunit I (1:1000; MitoSciences), mouse monoclonal anti-complex V subunit alpha (1:2000; MitoSciences), and mouse monoclonal anti-porin 31HL (1:3000; Mitosciences). All antibodies were diluted in Dako antibody diluent with background-reducing components (Dako). Immunohistochemical staining was performed as described previously (16).

3.6. Determination of the mtDNA copy number

The mtDNA copy number was determined by quantitative PCR as previously described (24).

3.7. Sequencing of *IDH1* and *IDH2*

A 592-bp fragment spanning the sequence encoding the catalytic domain of IDH1 including codon 132 was amplified using the primer pair IDH1f (3'-GAGCTCTATATGCCATCACTGC-5') and IDH1r (3'-TGTGTTGAGATGGACGCCTA-5'). The following cycle conditions were used for amplification of the IDH1 fragment: 96°C 10 sec, 35 x (96°C 2 sec, 70°C 30 sec), 72°C 30 sec. A 498-bp fragment spanning the sequence encoding the catalytic domain of IDH2 including codon 172 was amplified using the sense primer IDH2f (3'-GCTGCAGTGGGACCACTATT-5') and the antisense primer IDH2r (3'-AGGAAAGCCACGAGACAGAG-5'). The following cycle conditions were used for amplification of the IDH1 fragment: 95°C 10 sec, 35 x (96°C 5 sec, 65°C 30 sec), 72°C 30 sec. For the amplification of the IDH1 and IDH2 fragments the Gene Amp®Fast PCR Master Mix (2x) from Applied Biosystems was used. PCR products were treated with Exo SAP IT 500 (USB Corporation), and the CEQ DTCS Quickstart Kit (Beckman Coulter) was used for the sequencing reaction followed by separation with a CEQ 2000 DNA Analysis System (Beckman Coulter).

4. RESULTS

GNs (n = 7) displayed significantly lower total enzymatic activity of OXPHOS complexes compared to cortical kidney tissue (n = 14) and adrenal gland tissue (n = 4) (Table 1). A significant down-regulation of complex I was observed, with a residual enzyme activity of 12% compared with kidney control samples and 36% compared to normal adrenal gland tissue (Table 1).

Also the combined complex I + III activity was 60% lower in GNs than in normal kidney. The findings are supported by immunohistochemical (IHC) staining of complex I: GN tumor cells showed no staining of complex I, whereas normal non-tumor ganglionic cells present in the same samples stained positive for complex I (Figure 1).

Complex II activity was also significantly reduced by >90% in GNs in comparison to kidney and adrenal gland control tissue (Table 1). The low combined complex II + III activity in GNs is in agreement with the low complex II activity. Loss of complex II protein in GNs is confirmed by immunoblot analysis as well as by IHC (Figure 1 and 2). Complex III activity was 29% lower in GNs in comparison to kidney tissue and 43% lower in comparison to adrenal gland tissue. These findings are in agreement with WB analysis, which showed in 6 of 7 GNs no detectable complex III protein. The residual amount of complex III in case 7 can be explained by the relatively high number of ganglionic cells found in this sample by histology. IHC confirmed that GNs show very low levels of the complex III protein in comparison to normal ganglionic cell clusters (Figure 1).

Complex IV activity, too, was significantly diminished in GNs (reduced by 71% compared to cortical

Table 1. Enzymatic activities of the OXPHOS complexes in GNs, NBs, control kidneys and adrenal glands

Enzyme activity [mUnits/mg protein]	GN (n=7)	kidney (n=14)[5]	adrenal gland (n=4)	NB (n=14)[5]	[1]P value	[2]P value	[3]P value
Complex I	5 ± 2	43 ± 3	14 ± 3	10 ± 2	>0.0001	0.0334	0.073
Complex I + III	26 ± 2	69 ± 7	65 ± 16	47 ± 9	0.0003	0.0097	0.1177
Complex II	7 ± 4	128 ± 12	83 ± 10	15 ± 4	>0.0001	>0.0001	0.2360
Complex II + III	13 ± 3	53 ± 5	35 ± 4	19 ± 2	>0.0001	0.0019	0.0796
Complex III	98 ± 22	161 ± 10	173 ± 11	101 ± 9	0.0066	0.0377	0.8737
Complex IV	29 ± 10	99 ± 9	119 ± 36	28 ± 3	>0.0001	0.0138	0.9144
Complex V	3 ± 2	40 ± 4	14 ± 1	17 ± 7	>0.0001	0.0025	0.0143
Citrate synthase	38 ± 16	111 ± 8	242 ± 61	119 ± 8	0.0002	0.0024	>0.0001
mtDNA copy number	207 ± 34	2960 ± 460[4]		194 ± 22	0.0002		0.7646

Values are given in mean +/- SEM. GN: ganglioneuroma; NB: neuroblastoma; [1]P values for comparison of GNs and control kidneys; [2]P values for comparison of GNs and control adrenal tissues. [3]P values for comparison of GNs and NBs. [4]n=10. [5]Data have been previously published (12).

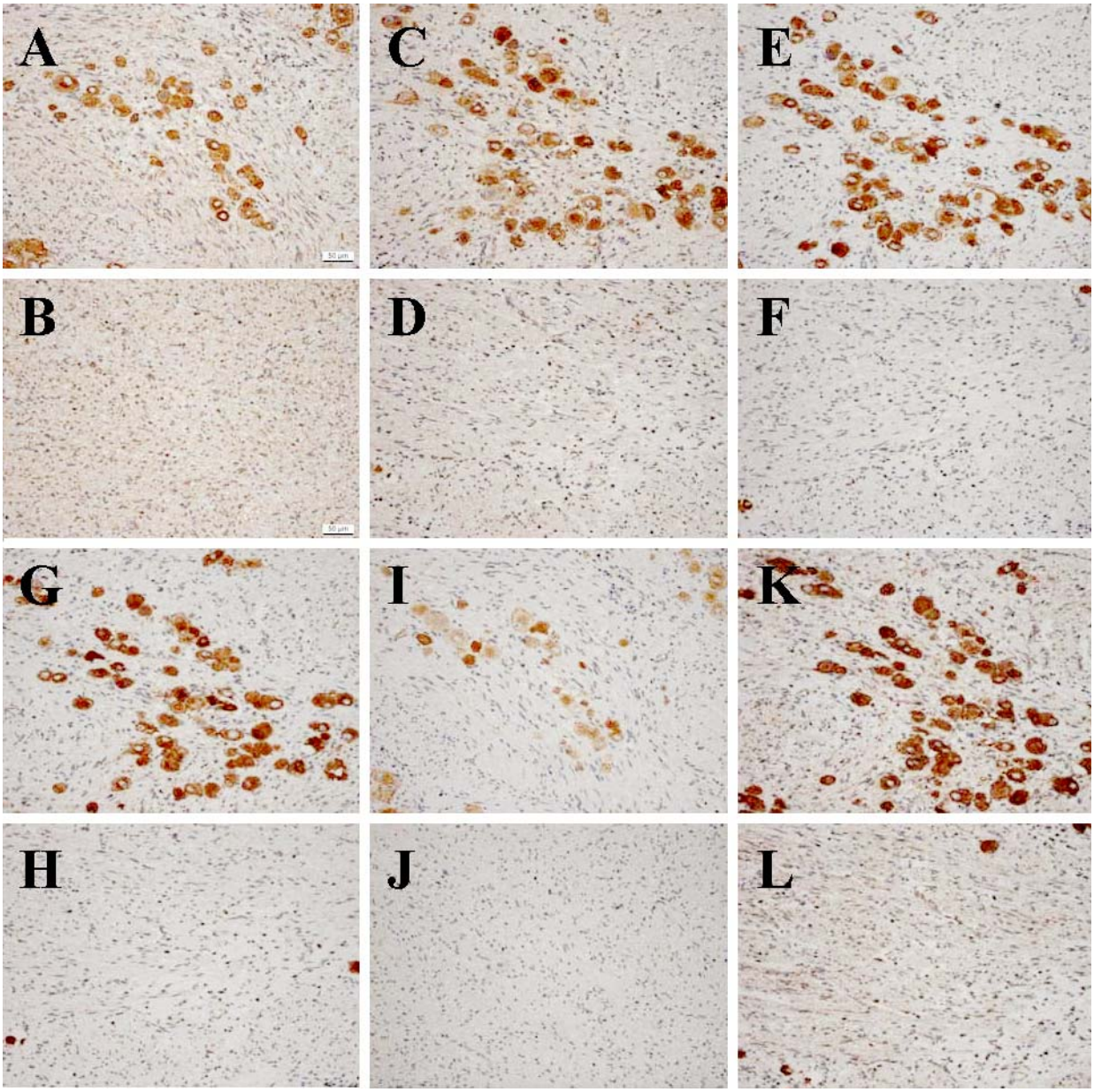

Figure 1. Immunohistochemical staining of GN tissue for OXPHOS complexes and porin. Porin (A, B); complex I subunit NDUFS4 (C, D); complex II subunit 70 kDa Fp (E, F); complex III subunit Core 2 protein (G,H); complex IV subunit I (I, J); and complex V subunit alpha (K,L). A, C, E, G, I, K: Area of the tumor tissue with non-tumorous ganglionic cells. B, D, F, H, J, L: region of the GN with pure tumor tissue of the same patient lacking ganglionic cells.

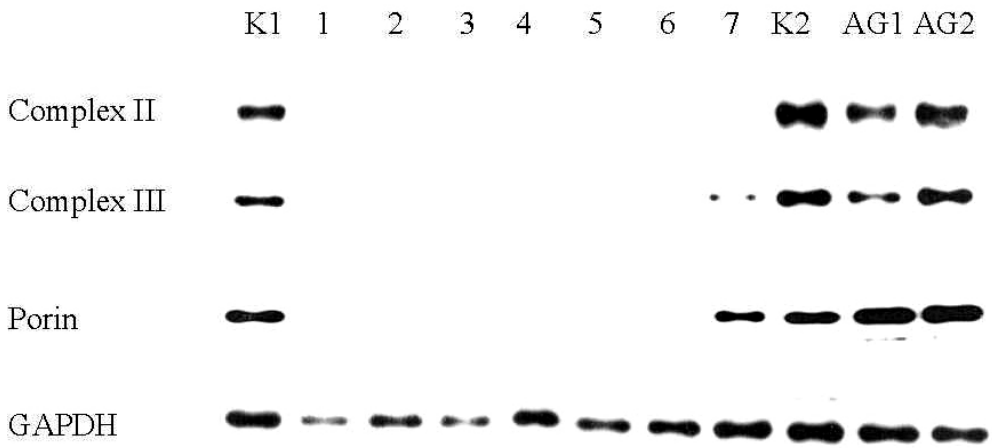

Figure 2. Western blot analysis of GNs, normal cortical kidney tissue and adrenal tissue samples. Complex II (SDHA; subunit 70 kDa Fp), complex III (subunit Core 2), porin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); lanes K1, K2: control cortical kidney; lanes 1 – 7: GN; lanes AG1, AG2: adrenal gland.

kidney tissue and by 76% compared to adrenal gland tissue). Again, no complex IV protein was detected by IHC, whereas normal ganglionic cells were positive for complex IV. Furthermore, the ATP synthase (complex V) activity in GNs was significantly lower (reduced by 92% in comparison to control kidneys, and by 79% in comparison to adrenal gland tissue; Table 1). In concordance with the absence of other OXPHOS complexes, complex V staining was negative in GN cells, whereas in ganglionic cells it was positive.

When the activities of the OXPHOS complexes of GNs were compared to the activities reported in NB (12), a trend of lower OXPHOS enzyme activities in GN compared to NB could be observed, although for most complexes the differences were not statistically significant.

Next we asked if the low activity of the OXPHOS complexes in GN is due to a reduction of mitochondrial mass. Therefore, the activity of citrate synthase, which is a citric acid cycle enzyme that is frequently used as marker for mitochondrial content, was measured. Citrate synthase activity in GN was 34% and 15% of the activity detected in the reference normal kidney and adrenal gland tissues, respectively (Table 1), suggesting reduced mitochondrial content. The activity of citrate synthase in GN was also significantly decreased in comparison to NB (12). Western blot analysis failed to detect porin, another marker protein for mitochondrial content, in 6 of the 7 GNs (Figure 2). The residual amount of porin observed in case #7 could be explained by the higher amount of ganglionic cells (5%) in this sample compared to the other tissues. Immunhistochemical staining also supports the lack of porin in tissue sections of GN samples, thereby confirming that GN cells are strikingly deficient in mitochondria (Figure 1).

In addition, mtDNA copy number was analyzed because it is also a marker for mitochondrial mass. GN showed a significant reduction in mtDNA copy number of 93% compared to unaffected cortical kidney tissue (Table 1). No statistically significant difference in mtDNA copy number was observed between GN (207 +/- 22) and NB (194 +/- 22).

Mutations in *IDH1* and *IDH2* have been shown to induce a glycolytic phenotype. To rule out that the loss of mitochondria is a consequence of IDH mutations we sequenced GN samples for the IDH mutations recently reported in other tumor types (25, 26). Notably, no mutations were detected at the two mutational hotspots, codon 132 of IDH1 and codon 172 of IDH2 as well as the surrounding gene regions encoding catalytic domains of IHD1 and IDH2.

5. DISCUSSION

Both types of heterogenous differentiated neuroblastic tumors, GN and NB, show a severe reduction in aerobic mitochondrial energy metabolism. The decrease of OXPHOS enzyme activity in GN can be explained as a result of reduced mtDNA copy number as well as mitochondrial mass. The consequences of the mtDNA depletion in GN are distinct from those observed in patients with mtDNA depletion syndrome, because the latter patients have normal or even compensatory high activities of nuclear-encoded enzymes such as complex II and citrate synthase (27). The initial event leading to down-regulation of OXPHOS might be the reduction of mtDNA copy number found in both NB and GN. The reduced mitochondrial mass observed only in GN could be a result of an event during tumor maturation from NB to GN. Tumor cells with a low mtDNA copy number may have an selective growth advantage. In ruling out that mutations in the glioma affected genes IDH1 and IDH2 (18) are associated with GN, we can also exclude that such genetic alterations were responsible for the mitochondrial defect in GN.

Low OXPHOS in tumor cells might result in the accumulation of upstream intermediates of the citric acid

cycle. In the case of GN due to mitochondrial deficiency, the tumor cells lack not only OXPHOS activity but also the citric acid cycle. Therefore, upstream cytosolic intermediates of glycolysis, fatty acid oxidation, and amino acid degradation will all accumulate. These intermediates might inhibit factors like hypoxia-inducible factor (HIF) hydroxylases, which are involved in the stabilization of HIF. It is assumed that HIF is the master regulator for the induction of the glycolytic switch in tumor cells. It has already been reported that fumarate, succinate and oxaloacetate are able to inhibit all three known HIF prolyl 4-hydroxylases (PHD) (28). Moreover, inactivation of PHD by a mutation in PHD2 was found in a patient with congenital erythromatosis and paraganglioma (29).

Furthermore, respiratory chain dysfunction in cancer cells may confer resistance to apoptotic cell death. Kwong *et al.* recently showed that both partial as well as complete loss of the respiratory chain protects cells from mitochondria-initiated apoptosis (30).

Mitochondrial DNA-deficient hepatoma cells are resistant to apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Dissipation of mitochondrial potential or cytochrome *c* translocation did not occur in rho^0 cells after TRAIL treatment (31, 32). The very low activity of OXPHOS complexes as well as mtDNA copy number might confer similar resistance to apoptosis in GN cells. This also would explain the fact that GN are resistant to chemo and radiation therapy (4).

Mutational inactivation of individual OXPHOS complex subunits (14-16), reduced activities of all OXPHOS complexes (13), or reduction of mitochondrial number (as observed in the present study) may all have the same consequence for tumorigenesis because each mechanism results in OXPHOS decline in tumor cells compared to unaffected tissues. In the case of GN, a factor influencing mitochondrial biogenesis may be responsible for the observed downregulation of mitochondrial mass. The mechanisms regulating mitochondrial number and mtDNA copy number in GN remain to be elucidated. One possible candidate is p53, which is present at very low levels in GN (33). p53 is an important regulator of mitochondrial energy metabolism and glycolysis (34). In this context, Lebedeva *et al.* showed recently that p53 null mice and human primary fibroblasts with knocked down p53 exhibit both mtDNA depletion and reduced mitochondrial mass (35).

In summary, the metabolic switch from OXPHOS to glycolysis seems to offer a selective advantage to various tumors. However, the molecular mechanisms leading to this alternative pathway of energy production differ between distinct tumor entities. For example, OXPHOS in pheochromocytomas, paragangliomas and oncocytic tumors, which frequently harbor mutations in mitochondrial-encoded OXPHOS enzyme subunits, can be decreased, even though only a single OXPHOS complex subunit is affected. In contrast, in the case of GN, the shift in cellular energy production is accomplished by a remarkable deficiency of mitochondria in GN tumor cells.

6. ACKNOWLEDGEMENTS

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