A mutation threshold distinguishes the anti-tumorigenic effects of the mitochondrial gene \textit{MTND1}, an oncojanus function

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Abstract

The oncogenic versus suppressor roles of mitochondrial genes has been long debated. Peculiar features of mitochondrial genetics such as hetero/homoplasmy and mutation threshold are seldom taken into account in this debate. Mitochondrial DNA (mtDNA) mutations generally have been claimed to be pro-tumorigenic, but they are also hallmarks of mostly benign oncocytic tumors where they help reduce adaptation to hypoxia by destabilizing hypoxia-inducible factor-1α (HIF1α). To determine the influence of a disassembling mtDNA mutation and its hetero/homoplasmy on tumorigenic and metastatic potential, we injected mice with tumor cells harboring different loads of the gene MTND1 m.3571insC. Cell cultures obtained from tumor xenografts were then analyzed to correlate energetic competence, apoptosis, α-ketoglutarate/succinate ratio and HIF1α stabilization with the mutation load. A threshold level for the anti-tumorigenic effect of MTND1 m.3571insC mutation was defined, above which tumor growth and invasiveness were reduced significantly.

Notably, HIF1α destabilization and downregulation of HIF1α-dependent genes occurred in cells and tumors lacking complex I, where there was an associated imbalance of α-ketoglutarate/succinate despite the presence of an actual hypoxic environment. These results strongly implicate mtDNA mutations as a cause of oncocytic transformation. Thus, the anti-tumorigenic and anti-metastatic effects of high loads of MTND1 m.3571insC, following complex I disassembly, defines a novel threshold-regulated class of cancer genes. We suggest these genes be termed oncojanus genes, to recognize their ability to contribute either oncogenic or suppressive functions in mitochondrial settings during tumorigenesis.
Introduction

Genes encoded by mitochondrial DNA (mtDNA) have long been suspected to be actively involved in tumorigenesis, when cells require high amounts of energy to grow and proliferate under few constraints. Although the association between somatic mtDNA mutations and cancer has been widely demonstrated, the mechanistic role played by these mutations is far from being elucidated.

The most credited hypothesis is that they may foster tumor progression in various ways such as through effects on regulation of apoptosis, hypoxia-inducible factor-1α (HIF1α) stabilization, reactive oxygen species (ROS) production and hence metastatic potential (1-4). A question still open in cancer biology concerns the oncogenic versus the oncosuppressor behavior of metabolic genes (5, 6), which incorporate both nuclear- and mtDNA-encoded respiratory complex subunits.

Enzymes such as succinate dehydrogenase (SDH) and fumarate hydratase (FH) are actively involved in tumorigenesis, through imbalance of the tricarboxylic acids (TCA) cycle and stabilization of HIF1α (7, 8). Similarly, respiratory complex I (CI) genes may be crucial in regulating cancer cell metabolism, since their function directly impinges on TCA. Alteration of oxidative metabolism resulting from mtDNA mutations may sustain, along with other causes, the triggering of the “Warburg effect” that characterizes cancer cells and permits a shift towards glycolysis, hence aiding tumor progression (9, 10). In this context, changes in the metabolic status of cancer cells are closely related to the degree of respiratory chain dysfunction, which in turn depends on both mtDNA mutation type (hampering function and/or assembly of complexes) and mutation load.

Reports that describe association of non-neutral mtDNA mutations with all types of cancers do not dwell on the peculiar features of mitochondrial genetics, namely the concept of homo- versus heteroplasmy, i.e. the coexistence of different mtDNA genotypes. Evaluation of the effects of mtDNA mutations must take into account the existence of a threshold above which a pathologic phenotype becomes evident, due to the physiological polyploidy of the mitochondrial chromosome, which has all too rarely been considered when dissecting the mechanisms underlying the metabolic
adaptation of cancer (3). In the vast majority of cases, clearly damaging mtDNA mutations described in tumors are reported as homoplasmic, suggesting that they may determine a pathologic phenotype despite an apparent positive selection (11-19).

A specific subset of tumors, namely oncocytic neoplasias, harbor high loads of damaging mtDNA mutations and yet retain, in most cases, a benign, low-proliferating, non-invasive behaviour (12-14, 20-23). A strong association between CI disruption and HIF1α destabilization has been demonstrated through increase of α-ketoglutarate (α-KG)/succinate (SA) ratio, which may be responsible for a higher affinity/activity of the prolyl-hydroxylase (PHD) that mediates HIF1α proteasomal-degradation. Such metabolic switch was suggested to explain why oncocytic tumors may escape malignancy (23). Thus, certain mtDNA mutations may indeed contribute to reduce tumor growth, depending on their effect on respiratory complexes assembly and their heteroplasmy threshold, which, to the best of our knowledge, has never been defined so far.

Here we demonstrate that high loads of a disruptive CI mutation frequently associated to human tumors (11, 12, 14, 15) hamper tumorigenic potential in vitro and in vivo. Threshold level for anti-tumorigenic effect of a mtDNA mutation in cancer, leading to oncocytic transformation, was determined, allowing us to define a novel type of tumor-implicated gene, namely the double-edged oncojanus.

**Materials and methods**

**Cell cultures and growth conditions**

Human thyroid carcinoma cell line XTC1 was derived from an oncocytic thyroid tumor (24). ZTC1 cells were a heteroplasmic clone obtained by serial dilution of XTC1 (22). Cybrids HXTC1 and HZTC1 were generated from human osteosarcoma 143B.TK cells and previously characterized (23). They will be renamed here as OS-93 and OS-85, respectively, the numbers indicating the percentage of MTND1 mutation load. A parental control cybrid (CC), previously characterized (23), homoplasmic wild-type for the m.3571insC and belonging to the same mtDNA haplogroup as OS-
93 and OS-85 was also used. OSC-83 and OSC-78 were derived from xenografts after *in vivo* injection of OS-85 cells. Cybrid cell lines were grown in DMEM, as previously described (23).

All cell lines were authenticated by mtDNA genotyping. Occurrence of the m.3571insC mutation and accurate quantification of heteroplasmy levels as previously described (25), was verified before and after explant as well as before each *in vitro* experiment. To the best of our knowledge no other cell line is known to harbor such mutation. Moreover, to authenticate cybrid cell lines, the occurrence of the known *TP53* mutation g.13055G>C harbored by the parental 143B.TK cells was verified (Supp. Fig.5). Coexistence of the *TP53* mutation along with the m.3571insC unequivocally identified cybrids, whereas thyroid cell lines harbored exclusively the m.3571insC mutation.

**Cell viability measurement**

Cells (4x10⁴) were incubated in glucose-free DMEM supplemented with 5mmol/L galactose, 5mmol/L Na-pyruvate and 10% FBS (DMEM-galactose). Viability was determined using the colorimetric sulforhodamine B assay (26).

**Complex I activity and ATP synthesis**

CI assembly and in-gel activity were determined after Blue-Native electrophoresis of isolated mitochondria, as previously described (27). The rate of mitochondrial ATP synthesis driven by CI and CII was performed in aliquots of digitonin-permeabilized cells and normalized on citrate synthase activity as previously described (28). Briefly, aliquots of cells (0.1–0.2 mg protein) were incubated with 5 mM malate plus 5 mM pyruvate (CI substrates) or with 10 mM succinate (CII substrate) plus 2 μg/ml rotenone. The reaction was started by addition of 0.2 mM ADP in the presence of luciferine/luciferase, and chemiluminescence was evaluated as a function of time with a luminometer. After addition of 10 μM oligomycin, the chemiluminescence signal was calibrated with an internal ATP standard.

**Soft agar assay**

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Anchorage-independent cell growth was determined in 0.33% agarose with a 0.5% agarose underlayer. Cell suspensions (1-3X10^4 cells) were plated in semisolid medium. Colonies were counted after 14 days at 40X magnification using an inverted microscope (Nikon Diaphot, Melville, NY).

**In vivo studies**

Cells (3x10^6) were suspended in 0.2ml sterile PBS and injected subcutaneously in different strains of immunodeficient mice: 4-7 week-old athymic Crl:CD-1-\textit{Foxn1}^{nu/nu} mice (referred to as nude mice, purchased from Charles River, Italy) and 23-31 week-old Rag2^{-/-};\gamma c^{-/-} on BALB/c background (breeders kindly given by Drs. T. Nomura and M. Ito, Central Institute for Experimental Animals, Kawasaki, Japan, then bred in our animal facilities under sterile conditions) (29). Experiments were authorized by the Institutional Review Board of the University of Bologna and performed according to Italian and European guidelines. Individually tagged virgin female mice (5-15 per experimental group) were used. Tumor growth was assessed with a caliper; volume was calculated as \[ \pi \sqrt{(a*b)^3/6}, \] where a=maximal tumor diameter, and b=tumor diameter perpendicular to a. Lungs were stained with black India ink and fixed in Fekete's solution to better outline metastases, which were then counted using a dissecting microscope.

**Immunohistochemistry**

Immunohistochemical staining (IHC) with antibodies against MTND6 CI subunit (Invitrogen, Milan, Italy) and HIF1\(\alpha\) (Upstate Biotech, Billerica, MA, USA) was performed, as previously reported (13).

**\(\alpha\)-ketoglutarate and succinate measurements**

Cells were incubated in DMEM with and without glutamine for 24h. Measurements of metabolites \(\alpha\)-KG and SA were performed essentially as previously described (23).
Western blotting

Total lysates (80μg) were separated by 10–12% SDS–PAGE and nitrocellulose membranes were incubated with antibody against actin (1:500, Santa Cruz Biotech, Santa Cruz, CA, USA), and HIF1α (1:1000, Bethyl Laboratories, Montgomery, TX, USA). Chemiluminescence signals were measured with a Kodak molecular imaging apparatus (Kodak, Rochester, NY, USA).

DNA extraction and DNA laddering analysis

DNA was kit-extracted from snap-frozen samples (Sigma-Aldrich, Milan, Italy) and 2μl were loaded on 1% agarose gel immediately after the extraction. Fragment sizing was determined through comparison with Gene Ruler 1kb DNA Ladder (Fermentas INC, Glen Burnie, MD, USA). Extractions from two distant tumor areas were performed for large xenografts to account for tissue heterogeneity.

MtDNA sequencing

Whole mtDNA resequencing was performed with MitoAll (Applied Biosystems, Foster City, CA, USA) as previously described (14) in order to verify that xenografts had not accumulated mutations apart from the m.3571insC.

Heteroplasmy evaluation

Mutant load for m.3571insC was determined using F-PCR according to previously optimized protocols for mutations in difficult sequence contexts such as in homopolymers (25). To ensure specificity of the mutant load evaluation, each analysis was performed in quadruplicate and all samples were run in the same reaction plate.

Electron microscopy (EM) and morphometric analysis


Xenograft biopsies were immediately collected and processed (14). Sections (1μm) were stained with 1% toluidine blue for morphology control and EM area selection. Thin sections were observed with JEM-1011 Transmission Electron Microscope (Jeol, Ltd). For each group three xenografts were analyzed, observing at least two different areas of each tumor to rule out intratumor heterogeneity. For morphometric analysis, 95 mitochondria per sample were measured on electron micrographs (magnification 15000X), and statistically analyzed using Olympus iTEM Imaging software.

**Pimonidazole staining**

Animals were injected intraperitoneally with 60mg/kg pimonidazole (Hypoxyprobe-1 Plus Kit, HPI, Burlington, MA) 3h prior to sacrifice. Xenografts were snap-frozen and cut in 10μm slices. Tissues were fixed for 20min with cold acetone, kept for 1h with PBS containing 5% FBS and incubated for 1h with FITC-MAb1 antibody (1:10). Fluorescence was visualized with a digital imaging system using an inverted epifluorescence microscope with 63X/1.4 oil objective (Nikon Eclipse Ti-U, Nikon, Japan) at 488nm. Images were captured with a back-illuminated Photometrics Cascade CCD camera system (Roper Scientific, Tucson, AZ, USA) and elaborated with Metamorph acquisition/analysis software (Universal Imaging Corp., Downingtown, PA, USA).

**RNA extraction and Real Time PCR analysis**

RNA was extracted from snap-frozen tissues with RNeasy Plus Mini kit (Qiagen, Milan, Italy) and cDNA was prepared using SuperScript III Reverse Transcriptase (Invitrogen, Milan, Italy) following manufacturers’ instructions. Gene expression levels of GLUT-1, VEGF-A and LDHA were measured with Real Time PCR using Sybr Green chemistry and 7500 Fast Real Time System (Applied Biosystems) through absolute quantification. Primer sequences and PCR conditions are available on request.

**Statistics**
SigmaStat 3.5 software was used for statistical analysis applying Student’s t-test unless otherwise indicated.

Results

High loads of the m.3571insC MTND1 mutation hamper tumor growth

We have previously generated and fully characterized a unique panel of human tumor cell lines harboring the m.3571insC mutation in MTND1 gene of CI, using two different tumor nuclear backgrounds and selecting different levels of heteroplasmy (23). We determined the precise load of mutant mtDNA by fluorescent PCR in all four cell lines used, namely the oncocytic thyroid carcinoma derived XTC1 (95.5%±0.35 mutation load) and ZTC1 (56.7%±0.7 mutation load) as well as osteosarcoma-derived cybrids OS-93 (92.8%±0.3 mutation load) and OS-85 (85.1%±0.8 mutation load). Anchorage-independent growth assay showed that nearly homoplasmic MTND1 mutant cells (XTC1 and OS-93) formed significantly fewer colonies compared with heteroplasmic and wild-type cells of the corresponding nuclear background. Interestingly, the heteroplasmic OS-85 cells showed the same in vitro growth pattern as the control cybrid (CC) (Fig. 1A and 1B).

To assess whether a high load of the m.3571insC mutation effectively hampered tumor growth in vivo, regardless of the nuclear context, we injected all five cell lines in two different immunodeficient mice models, namely nude and Rag2−/−;γc−/−. The growth patterns were coherent in both mice strains, suggesting that the difference in tumorigenic ability observed among cell lines was due to intrinsic properties and not to the residual immune response of mice. Overall, ZTC1 and OS-85 derived tumors grew significantly larger and faster than their respective high-load mutants XTC1 and OS-93 (Fig.1C and D and Supp. Fig.1). Similarly, CC cells grew in a completely superimposable fashion to OS-85 cells in vivo (not shown), concordantly with previously demonstrated analogous energetic efficiency (23). This finding prompted us to consider that OS-85 cells could be better controls than CC to take further on in subsequent analyses. OS-85 cells also showed a greater metastatic ability compared to OS-93 cells (Table 1). No other mutations apart
from the m.3571insC were detected in xenografts upon re-sequencing the whole mtDNA. Interestingly, the presence of high loads of mutant mtDNA strongly hampered the growth of the highly aggressive osteosarcoma. On the other hand, the least aggressive was the nearly homoplasmic mutant XTC1 cell line, which failed to grow in any injected animal (Fig.1C). Most importantly, high loads of a disruptive CI mutation were sufficient to reduce the tumorigenic potential of at least two different types of cancer, osteosarcoma and thyroid carcinoma, regardless of their intrinsic aggressive behavior.

**HIF1α destabilization, not apoptosis, contributes to tumor growth reduction**

We investigated the possible causes for the observed decrease in tumorigenic potential induced by the high m.3571insC mutation load. Apoptosis has been previously described to be differentially activated in homoplasmic versus heteroplasmic cells (3), yet typical apoptotic DNA laddering and nuclear chromatin condensation were observed exclusively in OS-85-derived tumors, regardless of their hosting mouse strain (Fig.2A and B), ruling out the role of apoptosis in the reduction of tumorigenic potential. On the basis of the occurrence of a chronic pseudo-normoxic condition in tumors bearing homoplasmic disruptive mtDNA mutations (23), we decided to investigate whether HIF1α destabilization was also induced in xenografts. Immunohistochemical (IHC) staining of CI MTND6 subunit was negative in OS-93-derived tumors but positive in most OS-85-derived masses. Correspondingly, HIF1α staining was negative wherever CI suffered from at least partial disassembly (Fig.2C) (23). Xenografts derived from OS-85 cells showed heterogeneous tumor volumes. Staining with hypoxic marker pimonidazole (30) revealed that small tumor masses displayed greater hypoxic areas compared to large ones, indicating that HIF1α stabilization was prevented in CI-deficient tumors despite the low-oxygen tension microenvironment in vivo (Fig.2D). Moreover, a significantly lower expression of HIF1α-responding genes, namely glucose transporter-1 (GLUT1), vascular endothelial growth factor A (VEGFA) and lactate dehydrogenase
A (LDHA), was observed in OS-93 xenografts (Fig.2E), suggesting that HIF1α destabilization had functional consequences on downstream gene expression.

**A mutation threshold must be reached to trigger tumor growth inhibition**

After detailed analysis of the individual growth curves of heteroplasmic-derived OS-85 osteosarcoma xenografts, we observed that only a few masses were markedly smaller than the majority (Fig.3A), which prompted us to quantify precisely the MTND1 mutation load in all developed tumors. A representative experiment is reported in figure 3B. Plotting heteroplasmy levels against nude mice tumor volumes indicated that only smaller OS-85-derived tumors maintained unaltered the original cell mutation load. On the contrary, all larger tumors underwent a shift toward the wild-type allele (Fig.3C), suggesting a purifying selection mechanism in order to overcome the damaging effect of mutations on tumor growth. A threshold for the m.3571insC mutation was hence defined between 81 and 83%, above which mitochondrial energetic impairment may not be sustained by cancer cells. In fact, tumor masses in which the genotype had partly reverted to wild-type below 81% presented CI subunit staining (Fig.2C). A load >83% was therefore sufficient to induce the same phenotype as a load close to homoplasmy, similar to that of OS-93-derived masses. A lower HIF1α protein expression level was observed in smaller OS-85-derived masses above threshold, confirming IHC data (Fig.3D) and the functional consequences on HIF1α-responding genes expression were comparable to those observed in Rag2⁻/⁻;γc⁻/⁻ (Supp. Fig.2). Interestingly, no tumor was observed to shift towards the mutant allele, suggesting that random drift may not be the mechanism determining a change in tumor genotype during progression, when non-neutral mutations are involved.

**Tumor growth reduction is closely linked to threshold-determined energetic impairment**

To assess the functional effects of the mutation threshold on cell phenotype and tumor growth, the smallest and largest tumors were utilized for further analyses, respectively bearing 83% (OST-83,
above threshold) and 78% (OST-78, below threshold) mutation load, originating from the very same culture batch of OS-85. Cell cultures were generated from xenografts (Fig.3A) and OSC-83 and OSC-78 cells were obtained (82.8%±1.5 and 78.3%±1.3 mutation load, respectively, Fig.3B) carrying identical heteroplasmy levels to those measured in xenografts. Viability of OSC-78 cells after 48h of incubation in galactose medium was significantly higher compared to OSC-83 and to the original OS-85 cells (Fig.4A). Therefore, only cells bearing a mutation load below the threshold were able to grow when forced to use oxidative phosphorylation solely for energy production. CI assembly and activity were markedly reduced in OSC-83, whereas no difference in complex II-driven ATP synthesis was observed, suggesting no alteration of the remaining oxidative phosphorylation (Fig.4B). Further, the α-KG/SA ratio was significantly higher in OSC-83 compared to OSC-78 both in presence and absence of glutamine (Fig.4C), demonstrating that CI disruption influenced the balance of these TCA cycle metabolites and that α-KG production in OSC-83 cells was glutaminolysis-independent. Instead of a variation of α-KG levels, such an increase was due to a relevant decrease in succinate (Supp. Table 1). Concordantly, stabilized HIF1α was detected exclusively in OSC-78 (Fig.4D). When OSC-78 and OSC-83 cells were re-injected in nude mice, OSC-78-derived tumors grew significantly larger than the high-load mutant OSC-83-derived tumors (Fig.5A), in agreement with the heteroplasmy threshold we have defined (Fig.5B). Finally, the contribution of ROS overproduction on HIF1α stabilization was ruled out in all clones analyzed. In fact, we failed to observe any significant difference in hydrogen peroxide and anion superoxide levels irrespective of the mutation load (Supp. Fig.3A and 3B).

*High loads of m.3571insC trigger oncocytic transformation*

The m.3571insC mutation is a hallmark of oncocytic tumors together with similar truncating mutations mainly occurring in homoplasmy (11-15, 21, 23, 31). In order to verify whether this mutation, when present above threshold, was sufficient to trigger oncocytic transformation, we examined xenografts by electron microscopy. We observed a mitochondria-rich phenotype only in
specimens above threshold level (Fig.6A). A striking resemblance to oncocytic tumors was evident in terms of mitochondrial number and swollen deranged organelle morphology, despite the non-epithelial origin of the osteosarcoma xenografts (Fig.6A, a and c). Morphometric analysis showed that both mitochondrial area and perimeter of OS-93 tumors were significantly increased compared to those of OS-85 tumors (Figure 6B and Figure 6C, respectively; P<1E-30). In fact, mean mitochondrial area was approximately (6±2.6)E5nm² in OS-93 derived tumors whereas it was approximately (1.8±1.1)E5nm² in OS-85 xenografts. Concordantly, mean mitochondrial perimeter was approximately 3.4±0.8μm and approximately 2±0.85μm respectively, showing that only OS-93 (above threshold) xenografts displayed mitochondria typical of oncocytic tumors.

The hypothesis that the same threshold of disruptive mtDNA mutations we have here defined accompanies oncocytic change in patients was supported by a re-analysis of over 100 cases from thyroid, pituitary and salivary gland, kidney and breast oncocytic neoplasms (12-14, 22, 23). In these tumors the heteroplasmy of the mtDNA mutations was evaluated. Most of the latter were found to affect CI assembly and presented a mutant load greater than 81%, confirming that this was the threshold needed to acquire an oncocytic phenotype (Supp. Fig.4).

Discussion

We have shown here that homoplasmy of a truncating mutation in MTND1 hampers the tumorigenic and metastatic potential of cancer cells in vivo. For the first time a precise threshold for such mutation, sufficient to increase α-KG/SA ratio, induce HIF1α destabilization and ultimately trigger growth arrest, has been identified.

Our proof for the principle regarding the anti-tumorigenic effect of mtDNA truncating mutations was obtained using a unique cell model (23), experimentally reinforced through re-injection of highly isogenic clones OSC-78 and -83, carrying a mutation load below and above threshold, respectively. In agreement with previous observations in patients (23), tumor xenografts bearing a mutation above threshold displayed pseudo-normoxia, showing HIF1α destabilization despite being...
truly hypoxic. HIF1α is considered to be one of the master regulators of the metabolic adaptation needed by cancer cells to progress to malignancy (7, 8, 32). Therefore this mechanism, tightly linked to the α-KG/SA imbalance as we have demonstrated, may be sufficient to drive the tumor into a ‘blind alley’ due to both respiratory impairment and lack of HIF1α-dependent glycolysis induction (22). Accumulation of NADH and inhibition of α-ketoglutarate dehydrogenase, rather than activation of glutaminolysis (10) may be the most plausible mechanism for α-KG/SA ratio increase as a consequence of CI disassembly. This imbalance may hence either boost the PHD affinity for molecular oxygen or diminish the availability of the main allosteric inhibitor of PHD, i.e. succinate, determining HIF1α destabilization even during hypoxia (33). In contrast with previous reports by Ishikawa et al. (1), who described a higher metastatic potential of cancer cells due to ROS-generating mtDNA mutations, we have ruled out the role of ROS in regulating HIF1α stabilization. This finding is concordant with our previous report (23) and is only apparently in disagreement with those of Ishikawa et al. (1), since the type of mtDNA mutation is different in our case. Oncocytic tumors display homoplasmic disassembling CI mutations, such as the m.3571insC here investigated, which may not be a ROS-generating mutation, if one considers that the main ROS production site might be lacking as a whole (23, 34-36). Moreover, the mutant load ought to be considered when analyzing functional effects of mtDNA mutations. The threshold model we propose here implies that, below threshold, CI is present and functioning correctly, in contrast to the case of a missense mutation leading to the coexistence of non-mutant along with mutant, ROS-producing CI.

The most important consideration comes from the strong association between homoplasmic disassembling mtDNA mutations and a mostly benign oncocytic tumor phenotype. Our studies in mice have shown that, upon trespassing the threshold, the mutation is sufficient to trigger oncocytic transformation, even in a non-epithelial cancer (Fig.6A). We have provided evidence that a single mtDNA mutation is able to generate a specific tumor phenotype, strengthening our hypothesis that
mtDNA mutations represent a secondary hit in cancer progression which may determine the oncocytic phenotype subsequently to primary oncogenic transformation (14, 21, 22). Patients with oncocytic tumors rarely present with highly aggressive, metastatic cancers. In the vast majority of cases, these tumors are surgically removed because of hindrance at the site of occurrence, such as in the case of oncocytic pituitary adenoma. In salivary glands, oncocytoma and Warthin tumors are considered benign and even, in some cases, non-neoplastic entities, similarly to oncocytic cells in Hashimoto’s thyroiditis (37). Kidney oncocytoma is usually considered benign and is only rarely metastatic. Interestingly, the main criterion used to distinguish renal oncocytoma from carcinoma during ultrasound scanning is absence of vascularization. Our findings provide an explanation for this clinical observation through HIF1\(\alpha\) destabilization and, in turn, lack of VEGF pathway activation. In thyroid, criteria for malignancy are independent of oncocytic transformation (37). In our long-standing experience on studying mtDNA sequence variation in all types of oncocytic tumors, the rare metastatic cancers encountered within the large sample set analyzed (over 150), were those not harboring mtDNA mutations. Therefore, mtDNA-determined oncocytic transformation appears to be functionally linked to a low tumorigenic and metastatic potential, which highlights the importance of homoplasy of mtDNA mutations as prognostic markers. Although warranting further investigation, our expression data on HIF1\(\alpha\)-responding genes clearly point to a down-modulation of some of the HIF1\(\alpha\) pathways as a strong candidate to re-direct the tumor fate, through the down-regulation of neovascularization and glycolytic metabolism. Induction of oncocytic transformation may therefore be envisioned as an approach to reduce tumor growth and abolish aggressive and metastatic potential.

Clinical applications may well arise from the translation of a genetic into a metabolic shift of the \(\alpha\)-KG/SA ratio towards \(\alpha\)-KG, which supports the use of cell-permeable \(\alpha\)-KG derivatives in anti-cancer therapy (38). Other strategies may impinge on CI disassembly in patients not harboring mtDNA mutations, degradation of assembly factors or even modulation of mitochondrial biogenesis to induce both the mutation and its accumulation. In fact, the phenomenon of homoplasmic shift
leading to a low-proliferating tumor deserves to be thoroughly understood to be exploited in pathology. In this context, a careful evaluation of the biogenesis and mitophagy pathways leading to mtDNA turnover will likely provide the mechanistic insights underlying the accumulation of non-neutral mtDNA mutations observed in patients.

The findings that tumors grew in mice only when a reversion towards wild-type occurred prompts a revision of the “Warburg effect”. It appeared that tumors were somehow forced to recover respiration to produce energy and, as our principle showed, stabilize HIF1α. Therefore, certain tumor stages appear to be respiration-dependent, most likely until more cell constraints have been removed and neovascularization has commenced together with nutrient influx to sustain a glycolytic shift. A model of waves of gene expression regulation to recover mitochondrial respiration during tumor progression has been recently proposed (10). According to such model, mitochondria revitalization is a necessary process in the transition from a highly glycolytic to a partial/enhanced oxidative phosphorylation cancer bioenergetic phenotype. Respiration recovery may well not occur in presence of a homoplasmic mtDNA truncating mutation, strengthening the anti-tumorigenic effect of CI disassembly.

Interestingly, the threshold we report here for the m.3571insC is analogous to what has been described in MERRF syndrome (Myoclonic Epilepsy and Ragged-Red Fibers). In patients with this mitochondrial disease, in fact, the threshold value for the causative m.8344A>G mutation has been described to be 80% (39), which further strengthens the hypothesis that 4/5 of mutant mitochondrial genomes in a cell may be sufficient to determine respiratory impairment.

In conclusion, our findings reveal a double-edged functional role for mitochondrial genes, closely correlated to a threshold effect. This property of such genes has allowed us to propose here the definition of oncojanus, indicating that, when mutated above a certain load, they determine an energetic impairment that prevents tumor growth.

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References


Table 1. Tumor and metastasis growth in immunodeficient mice.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mice</th>
<th>Tumor Incidence (%)</th>
<th>Tumor Incidence (%)</th>
<th>Lung metastases (median number, range)</th>
<th>Significance (Wilcoxon test)</th>
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<tr>
<td>OS-85 nude</td>
<td>15/15 (100%)</td>
<td>1/15 (7%)</td>
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<td>1/10 (10%)</td>
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<td>5/5 (100%)</td>
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<td>237-&gt;400</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>OS-93 Rag2&lt;sup&gt;−/−&lt;/sup&gt;;γ&lt;sub&gt;c&lt;/sub&gt;&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>5/5 (100%)</td>
<td>5/5 (100%)</td>
<td>43</td>
<td>4-89</td>
<td></td>
</tr>
<tr>
<td>OSC-78 nude</td>
<td>5/5 (100%)</td>
<td>5/5 (100%)</td>
<td>7</td>
<td>1-32</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>OSC-83 nude</td>
<td>5/5 (100%)</td>
<td>0/5 (0%)</td>
<td>0</td>
<td>0-0</td>
<td></td>
</tr>
<tr>
<td>ZTC1 nude</td>
<td>10/10 (100%)</td>
<td>0/10 (0%)</td>
<td>0</td>
<td>0-0</td>
<td>n.s.</td>
</tr>
<tr>
<td>XTC1 nude</td>
<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
<td>0</td>
<td>0-0</td>
<td></td>
</tr>
</tbody>
</table>

n.s.=not significant
Figure Legends

Figure 1. **In vitro** anchorage-independent growth and **in vivo** tumorigenic potential of cell lines. (A) Representative images of colony growth in soft-agar. Magnification 40X. (B) Quantification of colonies grown on soft-agar plates after 14 days; data are mean±S.D. (n=4, *P<0.05). Tumor growth generated from thyroid (C) and osteosarcoma (D) cell lines bearing different *MTND1* m.3571insC heteroplasmy levels injected in *nude* mice. Data are mean±S.E.M (n=3, five animals for each experiment; *P<0.05, **P<0.01).

Figure 2. Apoptosis and hypoxia in tumors. (A) DNA electrophoresis showing laddering exclusively in OS-85-derived tumors, regardless of hosting mouse strain. Individual tumors generated from OS-85 and OS-93 cells are indicated as OS-85(Tx) and OS-93(Tx) where ‘x’ is the number of the hosting mouse. (B) Nuclear chromatin condensation in high and low mutant load tumors. The black arrows indicate typical rose-shaped nuclei of apoptotic cells. (C) Representative IHC analysis on dissected tumor masses using antibodies against MTND6 and HIF1α. Magnification 100X. (D) Pimonidazole staining of representative OS-85-derived tumors of different sizes (T1: 3.02cm³ and 81% mutation load; T5: 0.31cm³ and 84% mutation load). Magnification 63X. Upper panels show overlay of the images captured at 488nm (lower panels) an in white light. (E) Gene expression levels of HIF1α-responding genes observed in OS-85 and OS-93 xenografts. Absolute mRNA quantification of GLUT-1, VEGF-A and LDHA in tumors explanted from Rag2⁻/⁻;γc⁻/⁻ mice is reported (*P<0.05, **P<0.01). Black bars indicate the average value.

Figure 3. **m.3571insC** threshold for anti-tumorigenic effect. (A) Growth curves of tumors generated after injection with OS-85 cells. One experiment on 5 nude mice representative of three independent experiments, for a total of 15 mice, is shown. Circles indicate the two tumor masses chosen for analysis reported in B. (B) Heteroplasmcy levels measured by fluorescent PCR. The arrows indicate the wild-type peaks in two tumors chosen for further analyses. (C) Scatter plot
showing that the m.3571insC mutation threshold for anti-tumorigenic effect lies between 81 and 83%. Every point is the mean value of a quadruplicate determination of mutation load ±S.D. in tumors obtained in nude mice at day 45 after injection. Tumors chosen for further analyses are circled. (D) Western blot analysis of HIF1α in OS-85 tumors explanted from representative nude mice. Actin was used as a loading control. AT: above threshold; BT: below threshold.

**Figure 4. Characterization of OSC-78 and OSC-83 cells.** (A) Viability of cell lines incubated for 48h in DMEM-galactose. Data represent mean±S.E.M. (n=5; *P<0.01). (B) CI in-gel activity. One representative experiment of three is shown. Mitochondrial ATP synthesis in digitonin-permeabilized cells driven by pyruvate/malate and succinate (CI and CII substrates, respectively) is reported. Rates, normalized for citrate synthase (CS) activity, are mean±S.D. (n=4; *P<0.05). (C) The ratio of α-KG and SA levels in presence (+Gln) and absence (-Gln) of glutamine was calculated by measurements for each metabolite in 3 different experiments (*P<0.05). (D) Western blot analysis of HIF1α in OSC-78 and OSC-83. Actin was used as a loading control. One representative experiment of three is shown.

**Figure 5. Heteroplasmy levels in OSC-78 and OSC-83 derived tumors.** (A) Tumor growth curves of OSC-78 and OSC-83 injected in nude mice (n=2, five animals for each experiment; *P<0.05). (B) Mutation loads in tumor masses were evaluated as reported in Materials and Methods. Data are mean±S.D. of at least four measurements.

**Figure 6. Oncoytic phenotype in osteosarcoma harboring high loads of m.3571insC.** (A) Electron microscopy of representative nude mice xenografts harboring m.3571insC above [OS-93(T3)] (a, c) and below [OS-85(T11)] (b, d) the threshold defined. Numerous, large mitochondria displaying clear matrix and almost total loss of cristae are present in (a) and clearly visible at the higher magnification of the inset (c). A heterogeneous population of mitochondria mostly with
darker matrix and normal cristae are shown in (b) and clearly evident at the higher magnification of the inset (d). Morphometric analysis showing the distribution of mitochondrial area (B) and perimeter (C) in OS-93 (above threshold) and OS-85 (below threshold) xenografts (n=95).
**A**

Electrophoretic gel images showing the expression of MTND6 HIF1α in different cell lines: OS-93(T3), OS-93(T11), OS-85(T1), OS-85(T5).

**B**

Cell images with arrows pointing to OS-85(T11) and OS-93(T3).

**C**

Microscopic images of MTND6 and HIF1α expression in OS-93(T3) cells.

**D**

Immunofluorescent images of GLUT-1, VEGF-A, and LDHA expression in OS-85(T5) and OS-85(T1) cells.

**E**

Graph showing the absolute mRNA quantification (a.u.) of GLUT-1, VEGF-A, and LDHA in OS-85 and OS-93 cells. The graph includes asterisks indicating statistical significance (p < 0.05 or p < 0.01).

*Graphical data and images are not transcribed textually.*
A.

Days after OS-85 cells injection

B.

Homopolymer length

C.

Tumor volume (cm³)

OS-85-derived tumors >1cm³
OS-85-derived tumors <1cm³
OS-93-derived tumors

D.

Mr (kDa)

BT
AT

HIF1α Actin

3571insC MTND1 mutation load (%)
A mutation threshold distinguishes the anti-tumorigenic effects of the mitochondrial gene MTND1, an oncojanus function

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