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Increase in mitochondrial biogenesis, oxidative stress, and glycolysis in murine lymphomas

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Abstract

Lymphomas adapt to their environment by undergoing a complex series of biochemical changes that are currently not well understood. To better define these changes, we examined the gene expression and gene ontology profiles of thymic lymphomas from a commonly used model of carcinogenesis, the *p53*^{-/-} mouse. These tumors show a highly significant upregulation of mitochondrial biogenesis, mitochondrial protein translation, mtDNA copy number, reactive oxygen species, antioxidant defenses, proton transport, ATP synthesis, hypoxia response, and glycolysis, indicating a fundamental change in the bioenergetic profile of the transformed T cell. Our results suggest that T cell tumorigenesis involves a simultaneous upregulation of mitochondrial biogenesis, mitochondrial respiration, and glycolytic activity. These processes would allow cells to adapt to the stressful tumor environment by facilitating energy production and thereby promote tumor growth. Understanding these adaptations is likely to result in improved therapeutic strategies for this tumor type.

Keywords

Mitochondria; Reactive oxygen species; Glycolysis; Lymphoma; p53; c-myc; Free radicals

Introduction

The P53 tumor-suppressor protein has many essential functions in cell cycle regulation, DNA damage and repair, cell death, glycolysis, senescence, and genetic maintenance [1,2]. P53 is central to tumor prevention, a fact highlighted by the observation that at least 50% of all human tumors bear mutations in *p53* or in a regulatory protein within its pathway [1,3].

In mice, loss of *p53* leads predominantly to the development of aggressive, genomically unstable lymphomas [4–6]. Interestingly, mutations in *p53* are a well-established unfavorable prognostic factor for malignant human adult lymphomas [7]. Although the pathology of lymphomas in *p53*^{-/-} mice is well documented, the genetic and biochemical factors that synergize with the lack of *p53* to produce such tumors are poorly understood. Lymphomas

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from $p53^{-/-}$ mice accumulate clonal chromosomal aberrations, such as amplifications and translocations in specific chromosomes [6]. This implies that further genetic changes, in addition to the original mutation ($p53$ deletion), are involved in T cell lymphoma progression.

A common genomic alteration in mouse models of lymphomagenesis is amplification of chromosome 15 [8–11]. It follows that crucial genes in these chromosomes drive the tumorigenic process. Several reports have demonstrated that the pleiotropic protooncogene *c-myc*, located on mouse chromosome 15 and the syntenic human chromosome 8, is amplified and overexpressed in lymphomas [12–17].

c-MYC is upregulated in many human cancers (reviewed in [18]), and this activation, in combination with the functional loss of $p53$, is among the most frequently recorded genetic lesions in human neoplasia [19]. Although it is widely accepted that *c-MYC* drives tumorigenesis by promoting proliferation, genome instability, and self-renewal, there are other key pathways influenced by *c-MYC* that may be essential for tumor development and maintenance [20]. Recently, it has been reported that *c-MYC* can upregulate mitochondrial biogenesis and mitochondrial respiration in cancer cells [21,22].

Mitochondria are essential organelles and key integrators of metabolism. Mitochondria also play vital roles in cell death, senescence, and cell signaling pathways and hence critically influence cell fate decisions [23–26]. Mammalian mitochondria contain their own 16-kb chromosome, which encodes 13 polypeptides, 12S and 16S rDNAs, and 22 tRNAs required for mitochondrial function [27]. As a trade-off for ATP production, mitochondria consume most of the cellular oxygen and produce the majority of reactive oxygen species (ROS) [28, 29]. ROS have been implicated in the etiology of carcinogenesis via oxidative damage to cell macromolecules and through modulation of mitogenic signaling pathways [30]. Mitochondrial dysfunction is implicated in a range of age-related diseases, such as several neurodegenerative, cardiovascular, and muscular diseases, and in diabetes [23,31,32]. How mitochondrial functions are associated with cancer is a fundamental and complex issue in biomedicine that is not fully understood [33,34].

Cancer cells must adapt to and modify their environment to survive. It is well documented that solid tumors induce a program of adaptive responses to enable them to thrive in hypoxic environments by changing their metabolism, including upregulating glycolysis and releasing angiogenic factors [35–37]. The transcription factor hypoxia-inducible factor 1 (HIF-1) is a principal component of the hypoxia response in cancer cells [38], where it regulates angiogenesis and glycolytic gene expression and inhibits mitochondrial respiration through the upregulation of PDK1 [39,40].

The $p53^{-/-}$ mouse is one of the most widely used models of lymphomagenesis [4,5]. These mice die predominantly of precursor T cell lymphomas that show high penetrance, low latency, and high levels of genetic instability. The $p53^{-/-}$ mouse is therefore an interesting model for in-depth study of lymphomagenesis, malignization, and metastasis.

To identify the genetic and biochemical adaptations that control the metabolism of these tumors, we carried out whole-genome gene expression profiling on $p53^{-/-}$ thymic lymphomas in comparison with wild-type thymic control tissue. Using this approach, we identified 1090 genes that are differentially expressed at statistically significant levels. Novel algorithms applied to gene ontology (GO) analysis show that several pathways involved in ATPase-coupled proton transport and mitochondrial bioenergetics are significantly overrepresented in the tumor gene expression data. Our results demonstrate that these tumors show high levels of oxidative stress, increased mitochondrial mass and mtDNA content, increased mitochondrial respiration, and overexpression of genes involved in glycolysis and mitochondrial electron transport. These alterations are correlated with increased expression of *c-MYC*. Our data

strongly suggest that these lymphomas mount a compensatory response *in vivo* that facilitates tumor survival through the simultaneous genetic upregulation of mitochondrial bioenergetic pathways and glycolysis.

Materials and methods

Mice

All procedures were carried out in accordance with the approved animal care protocols at the Buck Institute. C57BL/6J *Trp53*^{-/-} mice (Stock No. 002101) were acquired from The Jackson Laboratory (Bar Harbor, ME, USA). The *Trp53* alleles were genotyped with primers IMR13, IMR14, IMR336, and IMR337, as recommended by The Jackson Laboratory. *p53*^{-/-} mice were monitored three times a week and were killed when they became ill. At necropsy, lymphoma samples were used for RNA, DNA, and protein isolation and histopathological analysis. Small pieces of lymphoma tissue and wild-type thymus gland were disaggregated through a 70-mm filter (Falcon, Becton–Dickinson) to obtain cell suspensions for FACS analysis, cell-line derivation, and cryogenic storage. Lymphoma cells were cultured in RPMI complete medium supplemented with 10% fetal calf serum (FCS), antibiotics, antimycotics, glutamine, and sodium pyruvate (all from Cellgro).

Measurement of oxygen consumption

Oxygen consumption was determined by high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). Thymocytes from wild-type mice and tumor cells (BK4 and BY5 cell lines) were counted and resuspended in Hanks' balanced salt solution plus 25 mM Hepes at 2×10^7 cells/ml. The measurements were taken at 37°C. The instrumental background flux was calculated as a linear function of O₂ concentration and the experimental data were corrected for the whole range of O₂ concentrations using DatLab software (Oroboros Instruments). The O₂ concentration at air saturation at 37°C and local barometric pressure (92.6 kPa) for the culture medium (Hanks' balanced salt solution) was 175.7 μ M (O₂ solubility factor 0.92). Oligomycin (160 ng/ml) was added to inhibit ATP synthesis; rotenone (1 μ M) and myxothiazol (2 μ M) were used to determine non-mitochondrial oxygen consumption; the uncoupler FCCP (0.25 μ M for thymocytes and 0.5 μ M for tumors) was added to study maximal activity of the electron transport chain.

Flow cytometry analysis of ROS

Thymocyte suspensions from wild-type mice or from *p53*^{-/-} tumors were plated in complete medium at 10^6 cells/ml. After 24 h, cells were incubated for 1 h in complete RPMI medium containing 2 μ M dihydroethidine, 2 μ M CM-H₂DCFDA, or 0.1 μ M MitoTracker green (Molecular Probes). After the cells were washed in PBS with 1% BSA, cellular levels of ROS and mitochondria were determined by flow cytometry in a Becton–Dickinson LSR cytometer, and data were analyzed using the WinMDI version 2.8 software package.

Western blot

Lysates of primary tumors and control thymuses in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris, pH 7.2, 1 mM EGTA, and protease inhibitors (Mini-complete; Roche)) were analyzed by Western blot using the following antibodies: monoclonal mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (RDI-TRK5G4-6C5; Research Diagnostics), anti- β -actin (clone AC-15; Sigma), anti-nitrotyrosine (487923; Calbiochem), anti-HSP70 (AB6535; Ab Biochem), anti-porin (VDAC; A31855), mouse monoclonal anti-COX Va and Vb (A21363 and A21349, respectively; Molecular Probes), and anti-c-MYC (ab17356; Abcam).

Immunofluorescence

Paraffin sections (5 μ m) from thymic lymphomas and control tissue were deparaffinized and incubated in sodium citrate, pH 6.0, for antigen retrieval. The sections were incubated with 1:250 anti-porin antibody (A31855; Molecular Probes), washed, mounted in Vecta-shield H-1200 (Vector Labs), and processed for immunofluorescence by standard methods. Immunofluorescence images were acquired with a Nikon 90i fluorescence microscope fitted with a 60 $\times$ objective and appropriate filters and analyzed with the ImageJ software package.

Analysis of mtDNA copy number by real-time PCR

DNA from frozen tumor and control thymic tissue was extracted with phenol:chloroform and quantified by Nanodrop. For each PCR, total DNA (100 ng) was added to the Power Sybr Mix (Applied Biosystems) with each primer at 400 nM: sense, 13597 5'-CCCAGCTACTACCATCATTCAAGT-3', and antisense, 13688 5'-GATGGTTTGGGAGATTGGTTGATG T-3' [41]. Mitochondrial DNA content was normalized to the copy number of the nuclear gene Jag2. Jag2 PCR was performed with the following primers: sense, 5'-TGGTCCACTGAGAGTTGCTG-3', and antisense, 5'-TACTGGAGCTAGCCCAGGAT-3'. Relative mitochondrial DNA content was determined by the $\Delta\Delta C_t$ method in an ABI Prism 7000 PCR apparatus (Perkin-Elmer). DNA melting curves were performed to ensure that a single PCR product was amplified.

Tumor cytogenetics and cell cycle profile

Metaphases were prepared by mincing extracted lymphoma tissue and incubating the tumor cell suspension in RPMI medium containing 10% FCS, antibiotics, and 0.1 μ g/ml colcemid for 2 h. The mitotically arrested cells were treated with 0.56% KCl for 30 min, fixed in methanol:acetic acid 3:1, and dropped onto clean microscope slides. Metaphases were hybridized with a Cy3-labeled chromosome 15 painting probe (Cambio, Ltd) as recommended by the manufacturer or stained with DAPI for chromosome number evaluation. At least 20 metaphases were analyzed per tumor. Fluorescence images were acquired with a Zeiss Axioplan2 microscope equipped with a 63 $\times$ planapo objective.

Cell cycle profile

Approximately 1×10^6 methanol:acetic acid-fixed tumor and control (bone marrow) cells were incubated with 10 μ M propidium iodide and 10 μ M RNase A for 1 h at 37°C and analyzed by flow cytometry using a Becton–Dickinson LSR cytometer and the CellQuest software package.

Gene expression, gene ontology, and statistical analysis

See the supplementary methods for a description of these procedures.

Results

Characterization of gene expression in mouse thymic lymphomas

To better understand the genetic and biochemical changes that take place during lymphomagenesis, we compared the gene expression profiles of nine independent *p53*^{-/-} thymic tumors with wild-type thymuses from 3-month-old mice. To establish which gene expression changes were statistically significant, we selected all genes with an estimated false discovery rate (FDR adjusted *p* value) below 5%, based on the Benjamini–Hochberg method [42].

Expression data from thymic lymphomas indicated that, compared with control thymic cell populations, 623 transcripts were significantly downregulated and 465 upregulated (Table S1). Thymic lymphomas thus present a highly altered and complex transcriptional profile, with 1090 differentially expressed genes. Many of these genes are involved in diverse but

interconnected pathways, including hypoxia responses, glycolysis, oxidative phosphorylation, mitochondrial metabolism, oxidative stress responses, and DNA replication and repair (Table S1).

To determine which pathways were over- or underrepresented significantly, we applied a novel statistical approach to GO analysis (see supplementary methods). GO results indicated significant differences for 61 biological processes (Table S2). Of the 33 overrepresented GO categories, 6 are implicated in mitochondrial proton transport and ATP production (Table 1).

Mitochondrial processes are upregulated in thymic lymphomas

Given this finding, and the fact that mitochondria are key integrators of energy metabolism, we reanalyzed the data to investigate whether thymic lymphomas present significant differences in mitochondrial gene expression. This analysis showed that 36 mitochondrial genes are significantly upregulated and 15 significantly downregulated (Table 2). Among the upregulated genes are five subunits of ATP synthase (1.5- to 4.73-fold), 11 proteins involved in electron transport (1.7- to 3.8-fold), and 8 involved in mitochondrial protein translation (1.5- to 2.7-fold) (Table 2). In addition to these genes, the expression profile shows a significant 2.3-fold upregulation of citrate synthase ($p = 0.001$) (Table S1), a commonly used marker for intact mitochondria.

To confirm the expression data, five wild-type and tumor samples were analyzed by Western blot for complex IV subunits Va and Vb, nuclear components of the COX complex, and porin, a major mitochondrial protein involved in mitochondrial import/export. The gene expression profile had indicated that mRNAs encoding these proteins are upregulated 2.3- to 3.1-fold. The Western blots confirmed that the expression of mitochondrial proteins is strongly induced in the thymic lymphomas (Fig. 1), as well as in a set of B and T human lymphomas (Supplementary Fig. 1). These data thus indicate a pronounced upregulation of several important mitochondrial proteins in thymic lymphomas, suggesting a significant modification of mitochondrial metabolism.

Mitochondrial respiration is greatly increased in thymic lymphomas

The increased mitochondrial gene expression in the thymic tumors indicates that there may be an increase in mitochondrial respiration. To study mitochondrial function, oxygen consumption and cellular respiration rates were determined by high-resolution respirometry (Fig. 2). Basal oxygen consumption in tumor cells was around eightfold higher than in thymocytes (compare Figs. 2A and 2B). Oxygen consumption was inhibited when oligomycin was added to prevent ATP synthesis, stimulated when oxidative phosphorylation was uncoupled by addition of FCCP, and almost completely inhibited when the electron transport chain was blocked with rotenone (which inhibits complex I) plus myxothiazol (which inhibits complex III). Oligomycin inhibits mitochondrial ATP synthesis, therefore respiration that is sensitive to oligomycin is due to ATP synthesis, whereas respiration that is insensitive to oligomycin is due to proton leak pathways through the mitochondrial inner membrane. There was no significant difference between the proportion of respiration to drive ATP synthesis and proton leak in thymocytes or tumor cells. In the presence of FCCP respiration represents the maximal capacity of substrate oxidation under the experimental conditions. The concentration of FCCP necessary to obtain maximal respiration in tumor cells was twice that in thymocytes. The maximal capacity of the BY5 tumor cells was higher than that of thymocytes or BK4 tumor cells but this difference was not statistically significant. The residual rate in the presence of rotenone plus myxothiazol represents the non-mitochondrial oxygen consumption. It was determined as $3.87 \pm 3.43\%$ of the basal respiration rate in tumor cells and $3.63 \pm 2.37\%$ in thymocytes and was subtracted from the oxygen consumption rates to assess mitochondrial respiration.

Glycolytic and hypoxia-induced genes are overexpressed in thymic lymphomas

Glycolytic energy production is a hallmark of solid tumors [37,43] and is known to be upregulated in hypoxic tumor cells because of the lack of oxygen for oxidative phosphorylation [35]. The gene expression analysis showed that several critical glycolytic genes are transcriptionally upregulated in thymic lymphomas. For example, muscle phosphofructokinase, a key glycolysis regulator, was significantly overexpressed (3.8-fold), as were aldolase 1, phosphoglycerate kinase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (2.5-, 3.9-, and 2.3-fold, respectively; Table S1). Western blot of thymic lymphoma samples confirmed GAPDH protein overexpression of about 2-fold ($p<0.01$; Figs. 3A and 3B). Thus several glycolytic enzymes are significantly upregulated in $p53^{-/-}$ thymic lymphomas.

One of the best characterized mechanisms of glycolysis upregulation is the hypoxia response through activation of HIF-1 [38]. Several HIF-1 targets are significantly upregulated in the thymic tumors, by 2.5- to 8-fold (Table S3). These include EGL nine homolog 3 (*Caenorhabditis elegans*), heme oxygenase (decycling) 1, transferrin receptor, and pyruvate dehydrogenase kinase isoenzyme 3 [38,39]. These data strongly suggest that HIF-1 is activated in thymic lymphoma, which could, in part, explain the activation of glycolytic enzymes.

Thymic lymphoma cells undergo endogenous oxidative stress and activate mitochondrial biogenesis

Because the gene expression profiling and GO analysis clearly uncovered alterations in genes involved in mitochondrial bioenergetics and oxidative stress (Tables 1 and 2), we tested the hypothesis that $p53$ -deficient thymic lymphomas were subject to endogenous oxidative stress. For this, we measured ROS levels in control thymocytes and $p53$ -deficient lymphomas by flow cytometry, using the peroxide marker dichlorofluorescein diacetate (DCFDA) and the superoxide marker dihydroethidine (DHE). Compared with controls, tumor thymocytes showed a three- to fourfold increase in DCFDA and DHE fluorescence (Figs. 4A and 4B), indicating that the lymphoma thymocytes are in a state of chronic oxidative stress, in agreement with previous reports [44–46].

Concurrent with the high ROS levels, mRNAs for several enzymes involved in oxidative stress detoxification were significantly increased in the tumors, including peroxiredoxin 4, peroxiredoxin 6, peroxiredoxin 6-like, and glutathione peroxidase 1 (Table S1). These data support the hypothesis that high endogenous ROS concentrations elicit a compensatory response by upregulating the expression of antioxidant proteins.

Most ROS are generated during mitochondrial electron transport [29]. Our findings that mitochondrial protein content, gene expression, and respiration are significantly increased in the thymic tumors (Table 1 and Figs. 1, 2B, and 2C) strongly suggest that mitochondrial biogenesis is enhanced. To assess this possibility we used the mitochondrial dye MitoTracker green in flow cytometry studies of lymphoma and normal thymic lymphocytes. MitoTracker green fluorescence was higher in tumor lymphocytes (Fig. 4C), indicating upregulation of mitochondrial content. These results were validated by estimating mitochondrial porin levels by Western blot and *in situ* immunofluorescence (Figs. 1 and 5, respectively).

To further confirm that lymphomas contain increased mitochondrial content we measured mtDNA copy number, as a surrogate marker of mitochondria, by real-time quantitative PCR of a 117-bp mtDNA fragment [41]. Input genomic DNA was normalized by amplification of the Jagged-2 gene on chromosome 14. Our results indicate a significant ($p=0.0011$) difference between the ΔC_t values that corresponds to a 2.3-fold increase in mitochondrial DNA content in these tumor samples (Fig. 6), in agreement with the Western, immunofluorescence, and gene

expression results. Collectively, these results show that mitochondrial biogenesis is potently activated in $p53^{-/-}$ thymic lymphomas.

Thymic lymphomas are highly aneuploid and show amplification of chromosome 15 and overexpression of c-MYC

Previous studies had indicated that murine $p53^{-/-}$ thymic lymphomas are commonly aneuploid [6,47,48]. To test if the tumors studied here are indeed aneuploid we prepared metaphase spreads directly from the tumor tissue and analyzed chromosome number by DAPI staining (Supplementary Fig. 2). Our results indicate that 10/11 tumors have a median chromosomal number >40 and have a significant (sixfold, $p<0.05$) increase in the intracell chromosomal number variation (Supplementary Fig. 2) compared to primary hematopoietic cells.

Several experimental models of murine lymphomagenesis show amplification of chromosome 15, suggesting that genes in this chromosome directly or indirectly drive tumorigenesis [8–11,13,16]. We assayed chromosome 15 copy number in $p53^{-/-}$ thymic lymphomas by fluorescence *in situ* hybridization (FISH) and found that chromosome 15 is frequently triploid in these tumors (Fig. 7A).

The proto-oncogenic transcription factor c-MYC is located on mouse chromosome 15 and human chromosome 8 and is frequently amplified in mouse and human lymphomas [8–11, 13,17,49,50]. Thus, c-MYC is a likely candidate for driving lymphomagenesis in cooperation with the lack of $p53$. Western blot analysis confirmed that thymic lymphomas upregulate c-MYC (Fig. 7B), in accordance with previous reports [6,9].

Discussion

Precursor T cell lymphoma is a consistent feature of the $p53^{-/-}$ mouse model. The transcriptional and biochemical characteristics that allow these tumors to thrive despite the aggressive tumor microenvironment are not well defined. Here, we have used gene expression profiling, GO analysis, and biochemical approaches to show that mitochondrial biogenesis, mitochondrial respiration, and glycolysis are increased simultaneously in $p53$ -deficient thymic lymphomas to allow their survival, development, and progression.

Energy generation is fundamental for tumor cell survival. Solid tumors are usually oxygen deprived, and their bioenergetic metabolism is consequently believed to depend on glycolysis [43]. Evidence for overexpression of glycolytic genes in cancer cells continues to accumulate [51–54]. Our analysis shows that the bioenergetic gene expression profile is highly altered in $p53$ -deficient thymic tumors and that these tumors show robust induction of key glycolytic enzymes (Fig. 3, Table S1).

Upregulation of glycolysis in hypoxic tumors is thought to be mediated by activation of HIF-1. Consistent with previous findings, our data show significant upregulation of HIF-1 targets in T cell lymphomas (Table S3) and support a model in which upregulation of glycolytic genes is mediated, in part, through the hypoxic response [38]. A pivotal role in the induction of glycolytic enzymes has also been demonstrated recently for c-MYC [55], and its overexpression in thymic lymphomas reported here suggests that c-MYC may contribute to the upregulation of glycolytic enzymes in these tumors. HIF-1 and c-MYC can act synergistically to promote lymphomagenesis, and this may be related to a cooperative upregulation of glycolytic genes [56,57].

However, our results also indicate that, in addition to glycolysis, thymic lymphomas activate mitochondrial biogenesis. These tumors exhibit increased expression of several genes involved in ATPase-coupled proton transport and ATP synthesis. Among these are the cytochrome c

oxidase (COX) subunits Va and Vb. COX is the terminal enzyme complex of the electron transport chain and oxidizes cytochrome *c* to generate ATP. Increased expression of COX Vb is thought to decrease apoptosis by sequestering cytochrome *c* in the mitochondria, thus decreasing the activation of caspase-3 and caspase-9 [58].

Importantly, we report that the respiration rate of thymic tumors was around eightfold that of control thymocytes, in agreement with the increase in mitochondrial content (Figs. 1, 4C, and 5) and gene expression data (Tables 1 and 2). Furthermore, we observed no significant functional differences between mitochondria within thymocytes and tumors, indicating that these tumor mitochondria are functionally competent. The percentage of respiration used for ATP synthesis is the coupling efficiency, and the value obtained in thymocytes and tumors (~80%) is similar to values found in a range of other cell types [59].

Upregulation of mitochondrial content and respiration rates could seem to be in conflict with the HIF-1 data, because HIF-1 has recently been shown to downregulate mitochondrial ATP production and oxygen consumption by inducing pyruvate dehydrogenase kinase 1, which phosphorylates and inhibits pyruvate dehydrogenase, leading to reduced levels of acetyl-CoA [39,40]. Thus our finding that mitochondrial biogenesis and oxygen consumption are activated simultaneously with significant activation of HIF-1 targets and glycolytic genes suggests that these two major biochemical pathways are not always mutually exclusive.

Increased mitochondrial biogenesis in both mouse and human lymphomas is also evidenced by the upregulation of porin (Figs. 1 and 5 and Supplementary Fig. 1), increased mitochondrial mass, and increased mtDNA content. Increased mtDNA correlates with the significant upregulation of endonuclease G and heme oxygenase-1 (HO-1) transcripts in lymphomas (Table 2). Endonuclease G is reported to generate the RNA primers required for mitochondrial DNA replication by DNA polymerase γ [60], and HO-1 expression is needed for maintenance of mitochondrial biogenesis upon exposure to doxorubicin [61].

The upregulation of mitochondrial biogenesis may be an additional survival strategy for this type of tumor [62]. However, surprisingly, the gene expression profile does not show a significant increase in any of the known genes that activate mitochondrial biogenesis directly, such as TFAM, PGC-1 α , PPARs, NRF-1, or NRF-2 (reviewed in [36]). Intriguingly, our results (Table S1) indicate a significant decrease in the expression of the proline-rich nuclear receptor coactivator 1, a PGC-1 related protein involved in the activation of NRF-1 and in mitochondrial biogenesis [63,64]. Hence, this study suggests that there may be other key factors involved in the upregulation of mitochondrial biogenesis in thymic lymphomas. One clear candidate is c-MYC, which is commonly overexpressed in human and murine lymphomas [6,9] and has been linked to mitochondrial biogenesis [21,22,40,65].

The biogenesis of mitochondria depends on the coordinated expression of nuclear and mitochondrial genomes. It is therefore not surprising that c-MYC could play a central role, because its target gene network is estimated to comprise about 15% of all protein-coding genes [18]. The overexpression of c-MYC has been shown to increase proliferation, ROS production, and genetic instability [20,21,65]. In addition, through its actions on mitochondrial content and glycolysis, c-MYC may provide the mechanisms by which tumors meet their energy requirements. This may explain the common upregulation of c-MYC in lymphomas and the synergism between the p53 mutations and the c-MYC overexpression/amplification in a variety of tumors [16,19,20,66,67].

Although the GO analysis demonstrated upregulation of several mitochondrial components, others were downregulated. One of the significantly downregulated mitochondrial genes is uncoupling protein 1 (UCP-1), which has recently been reported in normal thymocytes [68,

69] (Table 2). UCP-1 plays a crucial role in maintaining the proton leak across the mitochondrial inner membrane, resulting in adaptive thermogenesis in brown fat [70]. In addition to thermogenesis, UCP-1 may prevent mitochondrial oxidative stress by limiting the mitochondrial membrane potential [71]; however, these effects may be cell and context dependent [72]. Reduced UCP-1 mRNA levels may favor a higher rate of ROS production consistent with our results. However, the observed decreased UCP-1 mRNA levels do not result in a significant change in proton leak in the tumor cells (Fig. 2C), which suggests that thymic lymphoma cells increase mitochondrial respiration through a significant increase in mitochondrial content per cell rather than an increase in mitochondrial coupling.

Our results indicate that the increased levels of ROS in the lymphomas may be a consequence of increased mitochondrial number and/or mitochondrial dysfunction. ROS production may be selected for in the tumor cells: our working hypothesis is that the oxidative stress plays a positive role in lymphomagenesis, by contributing to mitogenic signaling, genetic variation, chromosomal instability, and tumor evolution rate [44,73,74]. We also propose that the notable increase in mitochondrial content and respiration, together with enhanced glycolytic gene transcription, is selected to provide the bioenergetic resources and apoptosis protection needed for tumor growth. We also propose that the upregulation of c-MYC may be an important mediator in both the activation of glycolysis and the mitochondrial biogenesis that results in increased ATP production in this tumor type. Agents that selectively interfere with these pathways, such as mitochondrial polymerase inhibitors, for example, could improve the effectiveness of current treatments for T cell lymphomas. Indeed, small-molecule CDK1 inhibitors have been successfully used recently for the treatment of c-MYC-dependent tumors in murine models [75] and have been proposed for the treatment of human malignancies that overexpress c-MYC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Western blot of the mitochondrial proteins (A and B) cytochrome *c* oxidase subunits Va and Vb and (C) porin (VDAC), demonstrating a significant increase in mitochondrial proteins in *p53*^{-/-} thymic lymphomas (*n*=5) versus wild-type thymic tissue (*n*=5). (D) β -Actin was used as a loading control.

Fig. 2. Oxygen consumption and cellular respiration rates in (A) thymocytes and (B) tumor cells. Representative traces of oxygen concentration (thin line) and cellular respiration (thick line) of thymocytes and tumor cells measured by high-resolution respirometry (Oxygraph-2k). Arrows indicate the time of addition of oligomycin (160 ng/ml), FCCP (0.25 μM for thymocytes and 0.5 μM for tumors), and rotenone (1 μM) plus myxothiazol (2 μM). (C) Mitochondrial respiration rates calculated from (A) and (B). White bars, thymocytes; gray bars, BK4 tumor cells; black bars, BY5 tumor cells. Non-mitochondrial oxygen consumption was measured after the addition of rotenone plus myxothiazol and was subtracted from the cellular respiration rates. Mitochondrial respiration rates are expressed as a percentage of basal

respiration. Respiration driving ATP synthesis was calculated as the mitochondrial rate sensitive to oligomycin and respiration driving proton leak as the mitochondrial rate insensitive to oligomycin. Uncoupled mitochondrial respiration was calculated after the addition of FCCP. Data are means $\pm$ SD ($n=2-4$). There were no significant differences between thymocytes and tumor cells for any of the data in (C).

Fig. 3. (A) Western blot of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in wild-type thymuses ($n=5$) and $p53^{-/-}$ thymic lymphomas ($n=5$), showing increased GAPDH levels in tumor samples. β -Actin was used as a loading control. (B) Quantification of the relative levels of GAPDH from (A). ** $p<0.01$.

Fig. 4. Representative flow cytometry charts of thymocytes from $p53^{-/-}$ thymic lymphomas and wild-type nontumor tissue, showing (A) an increase in peroxides, as measured by the fluorescence of oxidized DCFDA; (B) an increase in superoxide, as measured by the fluorescence of oxidized DHE products; and (C) an increase in thymocyte mitochondrial content, as measured by the fluorescence of MitoTracker green. Two representative experiments are shown of $n=4$ (A and B) or $n=3$ (C) thymic tumors examined. White histograms depict fluorescence of tumor thymocytes; gray histograms depict fluorescence of wild-type thymocytes.

Fig. 5. Photomicrographs showing representative immunofluorescence staining of porin in paraffin sections of tissue from (A) wild-type thymus and (B) $p53^{-/-}$ thymic lymphomas. Porin is used as an index of mitochondrial content. Nuclei are stained with DAPI (blue) and porin is stained red.

Fig. 6. Real-time PCR amplification curves for mitochondrial DNA from thymocytes from two wild-type animals and six p53^{-/-} thymic tumors. The amplification curves for wild-type thymocytes are displaced to the right, which corresponds to a higher C_t value, indicating fewer copies of mtDNA than in the tumor thymocytes.

Fig. 7.

(A) FISH of metaphase spreads from two independent thymic lymphomas hybridized with a Cy-3-labeled whole chromosome 15 painting probe (red signal) and counterstained with DAPI (blue), demonstrating frequent triploidy of chromosome 15 in these tumors. (B) Western blot of c-MYC in wild-type thymuses ($n=4$) and $p53^{-/-}$ thymic lymphomas ($n=5$), showing significantly elevated c-MYC expression in tumors. β -Actin was used as a loading control.

Table 1Gene expression ontologies for mitochondrial bioenergetics in mouse $p53^{-/-}$ thymic lymphomas

GO ID	T/C ratio	BH-adjusted <i>p</i> value	GO term
GO:0008553	1.16	0.001161006	Hydrogen-exporting ATPase activity
GO:0019867	1.15	0.001236526	Mitochondrial outer membrane
GO:0015986	1.22	0.001469855	ATP synthesis-coupled proton transport
GO:0016469	1.22	0.001615088	Proton-transporting two-sector ATPase complex
GO:0015078	1.15	0.00206569	Hydrogen ion transporter activity
GO:0015992	1.12	0.002256386	Proton transport

Gene ontology results from significantly overrepresented gene categories (terms) in thymic lymphomas from $p53^{-/-}$ mice. Only GO processes with an estimated FDR *p* value of <5%, adjusted for multiple testing based on the Benjamini–Hochberg method (BH), were considered for analysis. T/C ratio refers to the GO expression ratio between tumors ($n=9$) and control thymuses ($n=5$).

Table 2Mitochondrial gene expression results in *p53^{-/-}* thymic lymphomas

Gene	Gene name	T/C	FDR	Function
<i>Upregulated</i>				
NM_172264	Choline dehydrogenase	4.73	0.002	Alcohol metabolism
NM_007506	ATP synthase, H ⁺ -transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 1	1.66	0.002	ATP generation
NM_138597	ATP synthase, H ⁺ -transporting, mitochondrial F1 complex, O subunit	3.15	0.001	ATP generation
NM_026468	ATP synthase, H ⁺ -transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 2	3.47	0.001	ATP generation
NM_027862	ATP synthase, H ⁺ -transporting, mitochondrial F0 complex, subunit d	2.02	0.002	ATP generation
NM_025983	ATP synthase, H ⁺ -transporting, mitochondrial F1 complex, ε subunit	1.56	0.002	ATP generation
NM_013898	Translocase of inner mitochondrial membrane 8 homolog a (yeast)	2.66	0.000	Chaperone, mitochondrial import
NM_178639	Sideroflexin 5	2.30	0.002	Citrate transport
XM_128696	NADH dehydrogenase (ubiquinone) 1α, subcomplex 11	1.74	0.003	Electron transport
NM_025551	NADH dehydrogenase (ubiquinone) 1α, subcomplex 12	3.34	0.000	Electron transport
NM_007747	Cytochrome c oxidase, subunit Va	2.32	0.003	Electron transport
NM_025628	Cytochrome c oxidase, subunit VIIb polypeptide 1	1.71	0.005	Electron transport
NM_009942	Cytochrome c oxidase, subunit Vb	3.11	0.000	Electron transport
NM_007748	Cytochrome c oxidase, subunit VIa, polypeptide 1	3.18	0.000	Electron transport
XM_485640	COX18 cytochrome c oxidase assembly homolog (<i>Saccharomyces cerevisiae</i>)	3.06	0.000	Electron transport
NM_023172	NADH dehydrogenase (ubiquinone) 1β, subcomplex 9	3.84	0.000	Electron transport
NM_153064	NADH dehydrogenase (ubiquinone) Fe-S protein 2	2.46	0.000	Electron transport
NM_026703	NADH dehydrogenase (ubiquinone) 1α, subcomplex 8	2.47	0.001	Electron transport
NM_025567	Cytochrome c-1	3.12	0.002	Electron transport
NM_010911	Nitrogen fixation gene 1 (<i>S. cerevisiae</i>)	1.91	0.003	Fe-S cluster generation
NM_025650	Ubiquinol-cytochrome c reductase (6.4-kDa) subunit	2.53	0.003	Fe-S cluster generation
NM_011695	Voltage-dependent anion channel 2	2.52	0.001	Mitochondrial import/export
NM_007931	Endonuclease G	2.55	0.003	mtDNA replication
NM_016895	Adenylate kinase 2	2.22	0.004	Nucleotide metabolism
NM_007573	Complement component 1, q subcomponent binding protein	5.06	0.000	Nucleus-mitochondrion interactions
NM_133668	Solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 3	2.75	0.001	Phosphate transport
NM_013604	Metaxin 1	1.84	0.001	Protein transport
NM_025434	Mitochondrial ribosomal protein S28	1.86	0.003	Translation
NM_024227	Mitochondrial ribosomal protein L28	2.02	0.001	Translation
NM_024174	Mitochondrial ribosomal protein S23	1.51	0.002	Translation

Gene	Gene name	T/C	FDR	Function
NM_025537	Ts translation elongation factor, mitochondrial	2.15	0.001	Translation
NM_053159	Mitochondrial ribosomal protein L3	2.15	0.001	Translation
NM_138591	G elongation factor 1	2.71	0.002	Translation
NM_025553	Mitochondrial ribosomal protein L11	2.40	0.002	Translation
NM_026498	Mitochondrial ribosomal protein S11	1.82	0.002	Translation
NM_025544	Mitochondrial ribosomal protein S15	2.30	0.002	Translation
<i>Downregulated</i>				
NM_172961	4-Aminobutyrate aminotransferase	0.17	0.000	Amino acid metabolism
NM_008293	Hydroxysteroid dehydrogenase-1, $\delta<5>-3-\beta$	0.60	0.005	Biosynthesis of hormonal steroids
NM_007751	Cytochrome c oxidase, subunit VIIIb	0.03	0.000	Electron transport
NM_010887	NADH dehydrogenase (ubiquinone) Fe-S protein 4	0.52	0.004	Electron transport
NM_007381	Acetyl-coenzyme A dehydrogenase, long chain	0.38	0.002	Fatty acid metabolism
NM_019946	Microsomal glutathione S-transferase 1	0.40	0.003	Glutathione metabolism
NM_023644	Methylcrotonoyl-coenzyme A carboxylase 1 (α)	0.16	0.000	Leucine metabolism
NM_145567	3-Hydroxyisobutyrate dehydrogenase	0.32	0.002	Lipid transport and metabolism
NM_026405	RAB32, member RAS oncogene family	0.17	0.002	Mitochondrial fission and signaling
NM_133201	Mitofusin 2	0.62	0.005	Mitochondrial fusion and apoptosis
NM_008441	Kinesin family member 1B	0.25	0.000	Mitochondrial transport
NM_021028	Thymidine kinase 2, mitochondrial	0.37	0.000	Nucleotide metabolism
NM_007621	Carbonyl reductase 2	0.02	0.000	Oxidative stress
NM_173733	Sulfite oxidase	0.28	0.001	Oxidoreductase and sulfur metabolism
NM_009463	Uncoupling protein 1 (mitochondrial, proton carrier)	0.01	0.000	Uncoupling

Gene expression results for mitochondrial proteins in the thymic tumors. Only gene expression differences with an estimated FDR p value of $<5\%$, adjusted for multiple testing based on the Benjamini–Hochberg method, were considered for analysis. T/C refers to the gene expression ratio between thymic lymphomas ($n=9$) and control thymuses ($n=5$).