Mitochondrial Cytochrome B Gene Mutation Promotes Tumor Growth in Bladder Cancer

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Abstract

Mitochondria-encoded Cytochrome B (CYTB) gene mutations were reported in different cancers, but the effect of these mutations on cellular metabolism and growth is unknown. In a murine xenograft and human model of bladder cancer, we show the functional effect of overexpression of a 21-bp deletion mutation (mt) of CYTB. Overexpression of mtCYTB generated increased reactive oxygen species (ROS) accompanied by increased oxygen consumption and lactate production. MtCYTB overexpression induced significant tumor growth in vitro and in vivo by triggering rapid cell cycle progression through up-regulation of the nuclear factor-κB2 signaling pathway. Tumor-generated ROS induced in vitro lysis of normal splenocytes. Thus, we present physiologic and functional evidence for the role of a bona fide mitochondrial gene mutation in cancer. [Cancer Res 2008;68(3):700–6]

Introduction

Understanding the contribution of nuclear genes to the sequential development of cancer continuously opens up new avenues for disease management. However, we have just begun to explore the role of mitochondrial gene mutation in tumor development. Mitochondria are the key regulators of oxidative phosphorylation system (OXPHOS) generating cellular ATP. The OXPHOS system is composed of five complexes (I–V) that are assembled from multiple polypeptides, some encoded by mitochondrial DNA (mtDNA) and others by nuclear DNA (1). Among the five complexes, complex-III (mitochondrial b61 complex or Ubiquinol-Cytochrome c oxidoreductase) is a membrane-bound enzyme that catalyzes the transfer of electrons from Ubiquinol to Cytochrome c, coupling this process to the translocation of protons across the inner mitochondrial membrane (2). Only Cytochrome B (CYTB) is encoded by the mitochondrial genome and is fundamental for the assembly and function of complex-III, and together with Cytochrome c1 and iron-sulfur protein, it forms the catalytic core of the enzyme (2).

Complex-III deficiency due to nonsense, missense, or frameshift mutations in the CYTB gene have been reported in association with severe exercise intolerance, myopathy, encephalopathy, cardiomyopathy, septo-optic dysplasia, and multisystem disorders (3, 4). Although there are several reports of CYTB mutation in tumors of different anatomic origin (5–9), the functional effect of these mutations in tumor development is unknown. We previously reported mutations in a number of mitochondria-encoded genes in primary bladder cancers including a 21-bp deletion (from nucleotide position 15,642–15,662) of CYTB (6). Here, we examined the effect of this 21-bp deletion mutation in a model of bladder cancer. We hypothesized that the exogenously overexpressed mutant (mt) CYTB protein in mitochondria could contribute to enhanced tumor growth.

Materials and Methods

Cell lines and reagents. The MB49 cell line is a carcinogen-induced transitional cell carcinoma derived from C57BL/6 male mice and cultured as described (10). We procured Simian virus 40– immortalized human uroepithelial cells (SV-HUC-1) cells from American Type Culture Collection. We purchased 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) from Molecular Probes. Anti-mouse CD31, 24-well Matrigel invasion chamber were procured from BD PharMingen. Antibodies against matrix metalloproteinase (MMP)-2, ACTIN were procured from Santa Cruz Biotechnology. We purchased anti-Bcl-2, Bax, nuclear factor-κB (NF-κB; p100/p52), CyclinD1, Cdk4, and Cdk6 antibodies from Cell Signaling. Antimitochondrial complex-III core-2 antibody was purchased from Mitosciences. We obtained 6-amino-4-(4-phenoxypyphenylethylamino) quinazoline from EMD Biosciences, Inc. Vitamin C was purchased from Sigma. All secondary antibodies were procured from Jackson Immunoresearch. FuGene6 transfection reagents were procured from Roche. Tissue culture medium was procured from Mediatech, Inc. Thymidine was procured from Perkin-Elmer. We purchased 4- to 6-week-old female C57BL/6 mice from The Jackson Laboratory. All PCR primers, recombinant human Interleukin-2 (rhl-2), and antiynic antibody were purchased from Invitrogen Corporation.

CYTB deletion construct and transfection. Earlier, we reported a seven amino acid (21 bp) deletion in the mitochondria-encoded CYTB gene (nucleotide position 15,642–15,662) in primary bladder tumors (6). The CYTB gene was converted into nuclear format, and both wild-type and the mutant gene (with seven amino acid deletion) was synthesized using long-range gene synthesis (Genescript Corp.) as described earlier (11). The mutant and wild-type genes were then subcloned into Sall and NotI sites of the phosphorolylated cytomegalovirus (pCMV)/myc/mito plasmid. The resultant plasmids were resequenced using the ABI BigDye cycle sequencing kit (Applied Biosystems) for verification of the insert sequences.

In transfections, MB49 and HUC1 cells were transfected with mt and wild-type (wt) CYTB plasmids in the presence of the FuGene 6 transfection reagent. An empty pCMV/myc/mito vector was also used for mock transfection of MB49 cells. Stable clones were selected in the presence of G418 (800 μg/mL) and were confirmed to express mtCYTB or wtCYTB in mitochondria by Western blot analysis using the antiynic antibody.

Animal model and tumor growth analysis. For tumor growth, 1 × 10⁶ cells in 100 μL PBS were injected s.c. at the left flank of immunocompetent 4- to 6-week-old female C57BL/6 mice. All experiments were performed in accordance with the Johns Hopkins University Animal Care and Use Committee guideline. Each group contained at least 12 mice, and each experiment was repeated thrice. Tumor growth was monitored everyday, and mice showing signs of morbidity were immediately sacrificed according to University guidelines. Tumor volume was calculated with slide calipers using the following formula: V = (A × B²)/2, where V is volume (mm³), A is the long diameter (mm), and B is the short diameter.
**Results**

Increased tumor growth, invasion, and angiogenesis in *mtCYTB*–overexpressing MB49 cells. MB49 bladder cancer–derived...
cells were stably transfected with wtCYTB, mtCYTB, and empty constructs. Expression of the myc-tagged CYTB fusion protein in the mitochondria was confirmed (Fig. 1A). We performed 3H incorporation to estimate cell proliferation in these transfected cells. Thymidine uptake was significantly higher in the mtCYTB cells compared with the wtCYTB (37,309 ± 8,740 versus 19,201 ± 3,282; P < 0.001) or empty vector–transfected cells (37,309 ± 8,740 versus 17,913 ± 2,324; P < 0.001; Fig. 1B). Thymidine uptake also remained significantly higher (P < 0.001) in two other mtCYTB clones compared with the wtCYTB or empty vector–transfected cells (Supplementary Fig. S4A).

The number and size of soft agar colonies (>2 mm) was significantly higher in the mtCYTB compared with the wtCYTB (139 ± 16 versus 37 ± 9; P < 0.0001) or empty vector–transfected cells (139 ± 16 versus 28 ± 4; P < 0.0001; Fig. 1C). The number and size of soft agar colonies (>2 mm) was also significantly

Figure 2. In vivo tumor growth and angiogenesis in CYTB-transfected MB49 cells. A, compared with empty vector– or wtCYTB-transfected cells, tumor growth was significantly higher in mtCYTB group at each indicated time point (P < 0.001). Points, mean; bars, SE. B, representative photograph showing tumor growth at day 17 in different groups as indicated. C, compared with empty vector– or wtCYTB-transfected cells, MVD; determined by the number of CD31-positive endothelial cells was significantly higher in mtCYTB group (P < 0.001). *, P < 0.05 versus control. Each experiment was repeated twice.

Figure 3. ROS production and ROS-induced lysis of normal splenocytes in mtCYTB-transfected cells. A, ROS production was measured by flow cytometry using cell-permeable dye 2',7'-dichlorodihydrofluorescein diacetate. Percentage of positive cells was represented in the top right quadrant. Number of ROS producing cells was significantly higher in mtCYTB group compared with empty vector– or wtCYTB-transfected groups (P < 0.01). MB49 cells treated with 100 μmol/L H₂O₂ served as positive control. B, wild-type, mtCYTB, or empty vector–transfected MB49 cells (Effector) were cocultured with rhIL-2–activated normal splenocytes (Target) isolated from C57BL/6 mice (E:T ratio, 1:50) for 8 h in a tissue culture incubator. Splenocytes cultured alone served as controls. Significantly higher percentage of lysis (P < 0.001) of the activated splenocytes was achieved by mtCYTB-MB49 cells compared with empty vector– or wtCYTB-transfected cells. Each experiment was repeated twice.
compared with control (Fig. 2A and B). All mice were sacrificed on day 17 because of the large tumor burden, and tumor volume remained significantly higher in the mtCYTB group compared with wtCYTB (2,392 ± 182 versus 612 ± 92 mm³; P < 0.0001) or empty vector–transfected group (2,392 ± 182 versus 594 ± 54 mm³; P < 0.0001; Fig. 2A and B). Two other mutant clones used in this experiment also showed significantly higher tumor growth (P < 0.0001) at different time points compared with the controls (Supplementary Fig. S4D).

Increased angiogenesis is also a key feature of tumor progression. Therefore, we determined the distribution of blood vessels in fresh tumor tissues by immunohistochemistry using anti-CD31 antibody. Mean vessel density (MVD), determined by the number of CD31-positive endothelial cells, was significantly higher in mtCYTB compared with wtCYTB (48 ± 8 versus 12 ± 2; P < 0.0001) or empty vector–transfected cells (48 ± 8 versus 7 ± 2; P < 0.0001; Fig. 2C).

Increased ROS production and ROS-induced lysis of normal splenocytes in mtCYTB cells. It has been suggested that mtDNA mutations that inhibit OXPHOS and impede electron flow down the electron transfer chain could increase ROS production and contribute to cancer (4). Therefore, we examined ROS production by the stably transfected cells using the cell-permeable dye DCFH-DA (11). Significantly higher amount of ROS was produced by mtCYTB cells compared with wtCYTB (89 ± 6% versus 66 ± 7%; P < 0.02) or empty vector–transfected cells (89 ± 6% versus 61 ± 5%; Fig. 3A). It is known that ROS species are used by neutrophils, eosinophils, and macrophages to kill bacteria and also tumor cells (15). Because stably transfected mtCYTB cells produced significant amounts of ROS (Fig. 3A), we hypothesized that tumor-generated ROS might be able to induce apoptosis of the normal splenocytes. The highest percentage of lysis of normal splenocytes was achieved by mtCYTB cells compared with wtCYTB (47 ± 11 versus 19 ± 5; P < 0.0001) or empty vector–transfected cells (47 ± 11 versus 17 ± 3; P < 0.0001; Fig. 3B).

Altered metabolism in mtCYTB-expressing MB49 cells. A functional defect in the OXPHOS system may force cancer cells to use glycolytic pathways for ATP production when oxygen is limited (16). We determined cellular respiratory activity by assessing oxygen consumption and lactate production. Both oxygen consumption and lactate production significantly increased (P < 0.0001) in mtCYTB cells compared with wtCYTB or empty vector–transfected cells (Fig. 4A and B).

Expression of mtCYTB in MB49 cells altered apoptosis and cell cycle regulation. The ratio of Bcl-2:Bax initially determines the ability to trigger mitochondria-mediated apoptosis via the release of Cytochrome c and apoptosis-inducing factors (17, 18). Therefore, we examined the expression of Bcl-2 and Bax in the mitochondria of the stably transfected cells. By Western blot analysis, we detected a considerable increase in Bcl-2 and slight decrease of Bax expression in mtCYTB cells compared with wtCYTB or empty vector–transfected cells (Fig. 4C).

It has been suggested that cytosolic or nuclear H₂O₂ (ROS) can activate NFκB or Akt signaling, and resistance to apoptosis (4). NFκB is a family of transcription factors, which in mammalian cells consists of Rel (c-Rel), RelA (p65), RelB, p50/p105 (NFκB1), and p52/p100 (NFκB2; refs. 19, 20). It has been shown that NFκB can result in elevated expression of cell-cycle genes (such as Cyclin D1), inhibitors of apoptosis (such as Bcl2 and Bcl-xL), and genes involved in an invasion phenotype (such as MMP-2; ref. 20). In a recent study, overexpression of the NFκB2-p52 protected tumor cells from apoptosis, and increased processing of p100 to p52 was
mediated by Stat3 (21). Because mtCYTB-MB49 cells generated a large amount of ROS, we performed Western blot analysis on lysates prepared from cultured cells, using antibodies against NFκB2 (p100/p52), CyclinD1, Cdk4/6, and MMP-2. There was a marked shift of expression of active p52 from the latent reservoir of p100 NFκB2 in mtCYTB-MB49 cells compared with wtCYTB or empty vector–transfected cells (Fig. 5A). The expression of CyclinD1 and MMP-2 also increased in mtCYTB-MB49 cells compared with wtCYTB or empty vector–transfected cells (Fig. 5A and B). However, the expression level of Cdk4 and Cdk6 was similar in all the groups (Fig. 5B).

Inhibition of ROS or NFκB diminished in vitro tumor growth. To examine the effect of ROS inhibition on cell growth and proliferation, we cultured mtCYTB-MB49 cells in the presence of vitamin C (100 μmol/L) as described earlier (11). Interestingly, vitamin C treatment significantly inhibited ROS production (Fig. 5B; P < 0.001), in vitro proliferation (top), and expression of NFκB2 (P < 0.001; Fig. 5C, bottom) in mtCYTB-MB49 cells.

To further confirm the effects of increased NFκB signaling, we treated mtCYTB-MB49 cells with 6-amino-4-(4-phenoxyphenylethylamino) quinazoline (50 μmol/L) for 6 h followed by cell cycle analysis. Untreated mtCYTB cells served as a control. Considerable G0-G1 arrest (M1) was evident by 6 h in the treated cells (61% versus 32%) compared with the untreated cells.
in the treated mtCYTB-MB49 cells compared with the untreated mtCYTB-MB49 cells (61% versus 32%; Fig. 5D).

Increased proliferation, invasion, and anchorage-independent growth of normal SV-HUC-1 cells after forced overexpression of mtCYTB. To examine the effect of CYTB overexpression in normal human uroepithelial cells, immortalized human SV-HUC-1 uroepithelial cells were stably transfected with wtCYTB and mtCYTB constructs, and expression of the myc-tagged CYTB fusion protein in the mitochondria was confirmed (Fig. 6A). No CYTB-fusion protein was detected in the cytosol or nuclear fraction depleted in the mitochondria (data not shown). Thus, stably transfected HUC-1 cells exogenously overexpress wt or mtCYTB proteins in the mitochondria.

To estimate cell proliferation, we performed 3H incorporation in these transfected cells. Thymidine uptake was significantly higher in mtCYTB cells compared with wtCYTB-HUC-1 (34,583 ± 3,495 versus 2,0132 ± 2,198; P < 0.004) or empty vector–transfected cells (34,583 ± 3,495 versus 19,371 ± 1,867; P < 0.003; Fig. 6B). We observed soft agar colony formation (Fig. 6C) and invasion (Fig. 6D) only by mtCYTB-HUC-1 cells compared with wtCYTB-HUC-1 or empty vector–transfected cells.

We determined respiratory activity by assessing oxygen consumption and lactate production in the CYTB-transfected HUC-1 cells. Both oxygen consumption (Supplementary Fig. S1A) and lactate production (Supplementary Fig. S1B) were significantly increased (P < 0.001) in mtCYTB-HUC-1 cells compared with wtCYTB-HUC-1 or empty vector–transfected cells. ROS-producing cells were significantly higher in the mtCYTB group compared with wtCYTB (72 ± 6% versus 56 ± 4%; P < 0.03) or empty vector–transfected group (72 ± 6% versus 52 ± 6%; P < 0.03; Supplementary Fig. S1C).

Apoptosis and cell cycle deregulation in the CYTB-overexpressing SV-HUC-1 cells. We also examined the expression of Bcl-2, Bax, and Bad in the mitochondria of the CYTB-transfected HUC-1 cells. We observed a considerably increased expression ratio of Bcl-2:Bax in the mtCYTB-HUC-1 cells compared with wtCYTB-HUC-1 or empty vector–transfected cells (Supplementary Fig. S2A). In addition, a remaining piece of tumor tissue from the cancer initially found to harbor the CYTB mutation also showed an increased expression ratio of Bcl-2/Bcl-x/L:Bax/Bad ratio (Supplementary Fig. S3).

Because CYTB-overexpressing HUC-1 cells generated a large amount of ROS, we hypothesized possible activation of the NFκB pathway as observed in the murine model system. We performed Western blot analysis on lysates prepared from cultured cells using antibodies against NFκB (p100/p52), CyclinD1, and Cdk4/6. We observed increased expression of active NFκB-p52 in mtCYTB-HUC-1 cells compared with wtCYTB-HUC-1 or empty vector–transfected cells (Supplementary Fig. S2B). Expression of Cyclin D1 and Cdk4 also increased considerably in mtCYTB-HUC-1 cells compared with wtCYTB-HUC-1 or empty vector–transfected cells (Supplementary Fig. S2B). Expression level of Cdk6 was undetectable in control cells, however, detectable in CYTB-transfected cells with considerably higher expression in mtCYTB-HUC-1 cells (Supplementary Fig. S2B).

Discussion

Little is known about the role of mitochondrial gene mutation in cancer. Here, we show the effect of overexpression of a 21-bp deletion mutation of the CYTB gene in uroepithelial human and murine bladder cancer models. The parent cells do not have a CYTB gene mutation, and the expression level of endogenous wild-type CYTB protein was similar in all the cell types (data not shown).

The mtCYTB expression resulted in significant in vitro and in vivo tumor growth and an increased invasive phenotype (Figs. 1 and 2), a hallmark for tumor growth and progression as identified long ago in human tumors and cell lines. Increased tumor growth was accompanied by the Warburg effect with marked shifts toward glycolysis (Fig. 4) and a large increase in ROS production (Fig. 3). This effect is specific and not just due to forced expression of a mutant CYTB gene because overexpression of wtCYTB resulted in no effect compared with control vector. Recent studies (1, 11), have shown increased ROS production in prostate and head and neck cancer after overexpression of mutant ATP6 and ND2 genes, respectively. Interestingly, the large amount of ROS produced by the mtCYTB-MB49 cells killed normal splenocytes, yet this had no effect on the tumor cells. Control NIH3T3 cells produced lysis only at background level (data not shown). This result suggested that the neoplastic cells might have developed a mechanism for scavenging from the toxic effect of ROS. This increase in ROS production might activate NFκB and set up the cell for a further increase in cell cycle progression, invasion, and inhibition of apoptosis. This may also explain the observed increased expression of CyclinD1 and MMP-2 together with a higher expression ratio of Bel-2:Bax proteins. Inhibition of ROS production and NFκB activation in the mtCYTB cells significantly inhibited in vitro cell proliferation, suggesting an important role for the pathway in mediating their growth.

To examine the effect of this seven amino acid deletion mutation on normal bladder cells, mtCYTB as well as the wt version was overexpressed in immortalized human uroepithelial SV-HUC-1 cells. The human SV-HUC-1 cells are not tumorigenic, so we could not assess in vivo tumor growth in these cells. However, as an immortalized uroepithelial cell line, HUC-1 are ideal cells to study the effect of gene expression leading to malignant transformation in human bladder tumorigenesis. Like the murine MB49 model, mtCYTB overexpression in HUC-1 cells resulted in similar pattern of changes in cellular growth and metabolism, further supporting the contribution of CYTB mutation in tumor growth promotion.

In a recent study, mutant CYTB was also identified as a potent tumor-associated antigen in melanoma patients, able to be recognized by MHC class II–restricted CD4+ T cells (22). NFκB inhibitors have also proved effective in killing neoplastic cells and are progressing toward therapeutic clinical trials. It thus seems that tumor cells with increased mt mitochondria may harbor putative targets for a variety of new therapeutic approaches.

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