Identification of COX17 as a Therapeutic Target for Non-Small Cell Lung Cancer

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Abstract

We have been investigating gene expression profiles in non-small cell lung cancers (NSCLCs) to identify molecules involved in pulmonary carcinogenesis and select which genes or gene products might be useful as diagnostic markers or targets for new molecular therapies. Here we report evidence that the cytochrome c oxidase (CCO) assembly protein COX17 is a potential molecular target for treatment of lung cancers. By semiquantitative reverse transcription-PCR, we documented increased expression of COX17 in all of 8 primary NSCLCs and in 11 of 15 NSCLC cell lines examined, by comparison with normal lung tissue. Treatment of NSCLC cells with antisense S-oligonucleotides or vector-based small interfering RNAs of COX17 suppressed expression of COX17 and also the activity of CCO, and suppressed growth of the cancer cells. Because our data imply that up-regulation of COX17 function and increased CCO activity are frequent features of lung carcinogenesis, we suggest that selective suppression of components of the CCO complex might hold promise for development of a new strategy for treating lung cancers.

Introduction

Lung cancer is one of the most common cancers in the world. NSCLC is by far the most common form, accounting for ~80% of lung tumors (1). The overall 10-year survival rate remains as low as 10% despite recent advances in multimodality therapy, because the majority of NSCLCs are not diagnosed until advanced stages (2). Although chemotherapy regimens based on platinum are considered the reference standards for treatment of NSCLC, those drugs are able to extend survival of patients with advanced NSCLC only ~6 weeks (3). Numerous targeted therapies are being investigated for this disease, including tyrosine kinase inhibitors, but thus far promising results have been achieved in only a limited number of patients, and some recipients suffer severe adverse reactions (4). By analyzing genome-wide expression profiles of 37 primary NSCLCs on a cDNA microarray containing 23,040 genes, we have been attempting to identify more selective molecular targets for development of strategies for earlier diagnosis, treatment, and/or prevention of NSCLC (3). In the course of those experiments we identified COX17 as one of the genes that was frequently overexpressed in lung tumors.

COX17 is an M, 8,000 protein that is essential for assembly of functional CCO in yeast and for delivery of copper ions to the yeast mitochondrion for insertion into the enzyme (6). Mutations among the six conserved cysteines of COX17 lead to respiratory defects in Saccharomyces cerevisiae (7, 8). Although this small protein has been cloned or purified from mammals as well, including human, mouse, and pig, its function in cancer cells or even in normal mammalian somatic cells has not been established (8–10).

Here we report functional characterization of COX17 in human lung tumors and in metabolism of normal mammalian cells. Our results suggest that overexpression of COX17 plays a significant role in development/progression of lung cancer and that this molecule represents a potential target for development of novel therapeutic drugs.

Materials and Methods

Cell Lines. Fifteen human NSCLC cell lines were used in this study: A549, NCI-H23, NCI-H522, LC174, LC176, LC319, PC14, PC9, NCI-H1435, NCI-H1793, SK-LU-1, RERF-LC-AL, SK-MES-1, and NCI-H358. Human SAECs and NHDF cells were used as controls. All of the cancer cells were grown in monolayers in appropriate medium supplemented with 10% fetal bovine serum. SAECs and NHDF cells were grown in optimized medium (SAECs and FGM-2, respectively) purchased from Cambrex Bio Science Inc. (Walkersville, MD). Primary NSCLCs were obtained with informed consent from NSCLC patients, as described previously (5), and 8 samples were used in this study.

Selection of a Candidate Gene and Analysis by Semiquantitative RT-PCR. Using the gene expression profiles of 37 primary NSCLCs obtained previously by cDNA microarray analysis (5), we selected genes that showed levels of expression that were 5-fold higher than in normal lung in >50% of the tumors examined. The COX17 transcript was among them. To confirm overexpression of COX17 by semiquantitative RT-PCR, total RNA was extracted from cultured cells and clinical tissues using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s protocol. The RNA preparations were treated with DNAase I (Nippon Gene, Tokyo, Japan) and reverse-transcribed into single-stranded cDNAs using oligo(dT)12–18 primer with transcriptase II reverse transcriptase (Life Technologies, Inc.). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification by monitoring the β-2-microglobulin gene (B2M) as a quantitative control. The primer sets for amplification were B2M-F (5’-TTAGCGTGTGTCTCGGCTACT-3’), B2M-R (5’-TCATACGTTCTCACCCGCAG-3’) for B2M, and COX17-F (5’-GTTGCACTAATTTGAGCC-3’), COX17-R (5’-GAAGATCTTCCACTAGTAAATTG-3’) with primers for the COX17 gene. All of the reactions involved initial denaturation at 94°C for 2 min followed by 22 (for B2M) or 27 cycles (for COX17) of 94°C for 30 s, 58–62°C for 30 s, and 72°C for 45 s on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA).

AS S-Oligonucleotides. Two × 105 cells from NSCLC line A549 plated onto six-well dishes were transfected with synthetic S-oligonucleotides (0.2 μM) corresponding to the COX17 gene, using Lipofectamine reagent (40 μM; Invitrogen, Carlsbad, CA), and maintained for 2 days in medium containing 10% fetal bovine serum. Cell numbers and cell viability were evaluated, respectively, by Giemsa staining and MTT assay, each in triplicate. The sequences of the S-oligonucleotides were as follows: AS, 5’-TCTCTCTTTCGATGATA-3’ and R, 5’-ATAGTACGTCCTTCTCCT-3’. MTT assays were performed as described elsewhere (11).

RNAi Assay. We had established a vector-based RNAi system, pshiHXB3.X.0, to direct the synthesis of siRNAs in mammalian cells, as reported elsewhere (12). Ten μg of siRNA-expression vector was transfected using 30 μl of Lipofectamine 2000 (Invitrogen) into NSCLC cell lines, A549 and LC319. In this assay, >90% of the transfected cells expressed this synthetic...
siRNA, and endogenous expression of COX17 was effectively suppressed. The transfected cells were cultured for 5 days in the presence of appropriate concentrations of geneticin (G418). Cell numbers and viability were measured by Giemsa staining and MTT assay in triplicate. The target sequences of the synthetic oligonucleotides for RNAi were as follows: control1 (EGFP; gene, a mutant of Aequorea victoria GFP), 5'-CAAGCGACGACGCTTCCTC-3'; control2 (Luciferase: Photinus pyralis luciferase gene), 5'-CGTACGGG-GAATTCCCTGA-3'; and control3 (Scramble: Chloroplast Euglena gracilis gene coding for the 5S and 16S rRNA), 5'-GGCCGCTTTGTAGGATTCG-3'.

We had observed that the COX17 transcript was overexpressed on a panel of 15 NSCLC cell lines and 2 normal human cells including SAEC, and then confirmed its increased expression in all of the 8 primary tumors and in 11 of the 15 cell lines examined (Fig. 1).

Growth-Inhibitory Effect of AS S-Oligonucleotides and siRNA Designed to Reduce Expression of COX17 in NSCLC Cells. To assess whether COX17 plays a role in growth or survival of lung cancer cells, we synthesized a pair of R (control) and AS S-oligonucleotides corresponding to the COX17 sequence and transfected each of them into A549 cells, a line that had shown a high level of COX17 expression. Introduction of the AS S-oligonucleotide decreased the number of foci formed and also decreased cell viability, compared with the control nucleotide, suggesting that COX17 was essential to the growth and/or survival of these cancer cells (Fig. 2A). To confirm that the growth suppression by AS S-oligonucleotide was gene specific, we also synthesized two siRNA expression plasmids designed to suppress endogenous COX17 and three different controls (siRNAs for EGFP, Luciferase, or Scramble), and transfected each of them into A549 and LC319 cells. As shown in Fig. 2B, siRNA-2 significantly suppressed expression of COX17 in the transfected cells, but neither siRNA-1 nor the three control siRNAs had this effect. Growth and viability of these two NSCLC cell lines were, in consequence, reduced significantly in cells transfected with siRNA-2 (Fig. 2, B and C).

Localization of the Product of COX17 and Activity of CCO in Mammalian Cells. To investigate the subcellular localization of proteins encoded by the human orthologue of yeast COX17, we transfected COS-7 cells with plasmids that contained c-myc-His-epitope sequences (LDDESLIKQE-HHHHHH) at the COOH terminal of COX17 protein and transfecting these plasmids to COS-7 cells. Transiently transfected COS-7 cells replated on chamber slides were fixed with PBS containing 4% paraformaldehyde, then rendered permeable with PBS containing 0.1% Triton X-100 for 3 min at 4°C. Cells were covered with 2% BSA in PBS for 30 min at room temperature, to block nonspecific antibody-binding sites. Then the cells were incubated with a mouse anti-c-myc antibody diluted 1:800 in the blocking solution. The murine antibodies were stained with a goat antimouse secondary antibody conjugated to FITC and viewed with a microscope.

CCO Activity in Lung Cancer Cells. LC319 cells were separated into mitochondrial and other fractions using digitonin (Wako, Osaka, Japan). To confirm fractionation we used mouse monoclonal antibody to human mitochondria (MABI273; Chemicon, Temecula, CA) as a positive control (13). Cytochrome c (63 μM) in buffer [10 mM Tris, 0.2 mM EDTA, and 0.05% n-dodecyl-β-maltoside, (pH 7.6)] was incubated with 12.5 μM L(+)-ascorbic acid for 30 min at room temperature (18°C) to convert ferric cytochrome c to ferrous cytochrome c. Twenty μl of the 1 mg/ml mitochondrial protein solution was then added to 2 ml of the reaction mixture at 37°C. The reaction representing CCO activity was measured at 550 nm absorbance.

Results

Overexpression of COX17 in NSCLC Tissues and Cell Lines. We had observed that the COX17 transcript was overexpressed on a cDNA microarray by the majority of our primary NSCLC cell populations, all of which had been purified by laser-capture microdissection (5). We additionally examined expression of this gene using semiquantitative RT-PCR in 8 additional NSCLC tissues, and our panel of 15 NSCLC cell lines and 2 normal human cells including SAEC, and then confirmed its increased expression in all of the 8 primary tumors and in 11 of the 15 cell lines examined (Fig. 1).
Discussion

Although advances have been made in development of molecular-targeting drugs for cancer therapy, the ranges of tumor types that respond as well as the effectiveness of the treatments are still very limited. Hence, it is urgent to develop new anticancer agents that target molecules highly specific to malignant cells and are likely to cause minimal or no adverse reactions. To achieve the goal we need to identify molecules of which the physiological mechanisms are well defined (14). A powerful strategy toward these ends would combine screening of up-regulated genes in cancer cells on the basis of genetic information obtained on cDNA microarrays with high-throughput screening of their effect on cell growth by inducing loss-of-function phenotypes with AS S-oligonucleotides and/or RNAi systems (5, 15–17). By pursuing such a strategy, we have shown here that COX17 is not only frequently overexpressed in clinical NSCLC samples and cell lines derived from that kind of tumor but also that the gene product is indispensable for growth of NSCLC cells.

CCO, the terminal enzyme of the mitochondrial respiratory chain, contributes to the proton motive force that drives synthesis of ATP (18). This multisubunit enzyme is conserved from lower to higher eukaryotes and requires three copper atoms for proper function (19). A number of genes encode assembly factors to constitute CCO function, and some of them play roles in utilization of copper. Mutations of COX17, which contains six conserved cysteines, cause a respiratory defect in yeast (7, 8). Because this defect can be rescued by addition of exogenous copper, COX17 has been assumed to function in the copper-trafficking pathway of cytochrome oxidase. For example, COX17 may shuttle copper ions to the mitochondria and transfer...
copper to yeast Sco1, an inner mitochondrial membrane protein (an intermediate step in copper donation to cytochrome oxidase; Ref. 7), but this hypothetical interaction has not been proven in either yeast or mammals.

To elucidate the function of COX17 in mammals, we examined the subcellular localization of COX17 protein in COS-7 cells and found it mainly in mitochondria. To clarify whether native COX17 protein is required for CCO activity in human NSCLC cells, we transfected COX17-siRNA vectors to LC319 cells, a line in which the COX17 gene is highly expressed. In those lung cancer cells, endogenous COX17 expression was suppressed significantly by siRNA, and CCO activity was reduced. Our results suggest that COX17 is likely to have an important role in the mitochondrial respiratory chain in humans, being essential moreover for CCO activity and, when overexpressed, for growth/survival of NSCLC cells. These assumptions imply a possibility of designing new anticancer drugs that target COX17 to specifically inhibit mitochondrial CCO activity. Strategies focusing on abrogation of metabolic pathways, including the CCO complex, that are significantly activated in cancer cells could facilitate development of novel therapies for treatment-resistant, advanced NSCLCs.

References
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