Enolase-α Is Frequently Down-Regulated in Non-Small Cell Lung Cancer and Predicts Aggressive Biological Behavior

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ABSTRACT

Purpose: Enolase-α is a cytoplasmic glycolytic enzyme important in the formation of phosphoenolpyruvate. Enolase-α and c-myc binding protein (MBP-1) originate from a single gene through alternative use of translational starting sites. Both enolase-α and MBP-1 can bind to the P2 element in the c-myc promoter and compete with TATA-box binding protein (TBP) to suppress transcription of c-myc.

Experimental Design: To determine a potential role of enolase-α in vivo, we analyzed enolase-α expression in non-small cell lung cancer (NSCLC) tissues from 46 patients by Western blotting and immunohistochemical analysis.

Results: Twelve (26%) of the 46 tumors showed a significantly reduced enolase-α expression. Although no statistically significant association was observed between the down-regulation of enolase-α expression, or differentiation, the patients whose tumors showed reduced enolase-α expression had a significantly poorer overall survival compared with those without down-regulation of this molecule (P = 0.039).

Conclusions: Our results indicate down-regulation of enolase-α expression in non-small cell lung cancer and may play an important role in lung tumorigenesis.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death in the United States. More than 157,000 patients will die of the disease this year alone. Development of lung cancer is the result of an accumulation of molecular abnormalities that activate oncogenes and inactive tumor suppressor genes. Understanding the biology of the disease is critical to developing novel strategies for early detection, prevention, classification, and treatment. The c-myc proto-oncogene is a critical factor in controlling both cell proliferation and apoptosis (1–4). The c-myc oncogene is important in tumor progression in multiple tumor types, including NSCLC1 (2, 3). Several distinct mechanisms have been suggested as regulating c-myc expression (5). Because c-myc has multiple promoters, termed P0, P1, P2, and P3, regulation of its gene expression is complicated. Yet in normal and transformed cells, the majority of c-myc transcripts are initiated through the P2 promoter (3, 4).

MBP-1 is a Mr 37,000–38,000 protein that can bind just 5′ to the P2 TATA motif and decrease c-myc promoter activity in both human and mouse models (6–8). Both MBP-1 and TBP can bind to the minor groove of the c-myc P2 promoter, suggesting that MBP-1 may negatively regulate c-myc transcription by preventing transcription of a transcriptional initiation complex (8, 9). MBP-1 mRNA is identical to enolase-α mRNA, which encodes a polypeptide of about Mr 48,000 using an alternative translation site (GenBank accession nos.: M14328 and M55914). In vitro transcription and translation experiments show that the enolase-α transcript encodes 2 proteins, Mr 48,000 and 37,000, both of which have the ability to down-regulate c-myc expression (10, 11). To determine a role of enolase-α in lung tumorigenesis, we analyzed enolase-α protein expression in 46 primary NSCLC. We found that enolase-α expression was down-regulated in 26% of the primary NSCLC and that such down-regulation was associated with a poor clinical outcome, suggesting that enolase-α plays an important role in NSCLC.

MATERIALS AND METHODS

Study Population. Surgically resected primary tumors and corresponding normal lung tissues were obtained from the Department of Pathology at The University of Texas M. D. Anderson Cancer Center after patients’ consent. These specimens were collected between 1995 and 1998, and follow-up information was obtained from the Tumor Registry at the M. D. Anderson Cancer Center. All of the patients were treated by surgery at the time of diagnosis. The primary tumor specimens consisted of 20 adenocarcinomas, 19 squamous cell carcinomas, and 7 samples of other cell types. The study population consisted of 24 males and 22 females, with a mean age of 64.1 ± 10.83 years. Other clinical characteristics are shown in Table 1.

Protein Extraction. The samples consisted of 46 paired normal/tumor tissues from patients with lung cancer. Briefly, samples were weighed and diced into small pieces with a clean
This antibody, donated by Dr. E. F. Plow, does not cross-react dry milk to reduce background.

PAGE gel under reducing conditions. Separated proteins on the membranes (Amersham Pharmacia Biotechnology, Arlington Heights, IL). Films (Amersham Pharmacia Biotechnology). Each signal was detected by enhanced chemiluminescence on Hyperfilm ECL (Abbott Laboratories, Irvine, CA) solutions were used serially, and the slides were counterstained with hematoxylin.

In univariate analysis, independent sample t tests and χ² tests were used for continuous and categorical variables, respectively. Kaplan-Meier analysis was performed to estimate a survival function over time for individual covariates. The log-rank test was used to compare patient survival time between groups. All of the statistical tests were two-sided. P < 0.05 was considered to be statistically significant.

RESULTS

Enolase-α Is Frequently Down-Regulated in NSCLC.

We analyzed the expression level of enolase-α from 46 normal/cancer pairs from patients with NSCLC through Western blotting. Although 42 (91.3%) of 46 of the tumors showed some degree of reduced enolase-α expression, we used a more stringent cutoff (a 2-fold reduction) to offset experimental variations (Fig. 1). Twelve (26.1%) of the tumors were assigned to the down-regulation group according to this criterion. We performed further IHC to determine enolase-α expression status at the cellular level. In normal lung tissues, IHC showed a ubiquitous cytoplasmic and membranous staining, including staining of the bronchial epithelium and alveolar wall (Fig. 2A). Nuclear staining also was observed in bronchial epithelial cells, type I and type II alveolar cells, and endothelial cells, consistent with a recent report (10). Endothelial cells showed a strong cytoplasmic and nuclear staining with the anti-enolase-α antibody. Lymphocytes, however, were barely stained with this antibody. In contrast, tumors exhibiting down-regulation of enolase-α exhibited lack of antibody staining, consistent with Western blotting analysis (Fig. 2 B and C).

Enolase-α Expression and Clinical/Pathological Parameters. We analyzed clinical and pathological parameters according to enolase-α expression status. In univariate analysis, we found no age and gender differences among patients with down-regulation and those without down-regulation. In addition, no significant associations were found between down-regulation of enolase-α (ratio, ≥2.0).
regulation of enolase-α and pathological stage, tumor histology, or the degree of differentiation. Although the difference was not statistically significant, patients whose tumors showed down-regulation of enolase-α bore larger tumors at the time of surgery (8.3 ± 8.58 cm) than did patients without down-regulation (4.8 ± 2.72 cm; Table 1).

**Down-Regulation of Enolase-α and Clinical Outcome.**

At last follow-up, 29 of the 46 patients had died, and 17 were still alive. Among the living patients, the median follow-up duration was 5.7 years. During the same follow-up period, 9 (75.0%) of the 12 patients whose tumors showed loss of enolase-α expression died, compared with 20 (59%) of the 34 patients whose tumors retained enolase-α expression.

Kaplan-Meier estimates were used to examine the relationship between down-regulation of enolase-α and patients’ overall survival. The median survival of patients whose tumors showed down-regulation of enolase-α was only 6 months (95% CI, 0.00–19.9 months), whereas the median survival of those whose tumors showed no down-regulation was 43 months (95% CI, 8.1–77.7 months; P = 0.0398 by log-rank test; Fig. 3).

**DISCUSSION**

Enolases have been characterized as highly conserved cytoplasmic glycolytic enzymes that catalyze the formation of phosphoenolpyruvate from 2-phosphoglycerate, the second of the two high-energy intermediates that generate ATP in glycolysis (12). Three isoforms of enolase have been identified and named as enolase-α, -β, and -γ. Enolase-α expression has been detected in almost all of adult tissues, whereas enolase-β is expressed predominantly in muscle and enolase-γ is detected only in nerve tissues (13, 14). These three isoforms may exist as either homodimers or heterodimers.

Diverse functions of enolase-α have been reported in the ecosystem. It has been identified as a heat shock protein in yeast (15), an immunodominant antigen in Candida albicans (16), and toxin B in Clostridium difficile (17). It also known as a component of the centrosome in HeLa cells (18) and as a molecule associated with connective tissue disorders (19). Enolase-α is considered to have potential roles in tumorigenesis. Tumor cells possess higher metabolic rate than surrounding normal tissues, and enolases, the representative glycolytic enzyme, are an important factor in cell metabolism. There is evidence to suggest that enolase-α may involve cancer invasion and metastasis. Enolase-α has a COOH terminus that spans plasma membrane. With this domain, monocytes, neutrophils, and some cancer cell lines, such as U937, are capable of binding with, activating, and stabilizing plasminogen. By doing so, they have the capacity to clear a path for themselves through the macromolecular barriers of basement membrane and other extracellular matrix (20).

Additionally the gene encoding enolase-α (ENO-1) maps to a region of human chromosome 1(1p35-p36) that is often deleted in several types of human malignancies, including neuroblastoma, melanoma, pheochromocytoma, and carcinoma of the breast and liver (21). Furthermore, as described earlier, the capability of enolase-α to compete with TBP to repress transcription of oncogene c-myc suggests a potential mechanism for how enolase-α may be involved in tumorigenesis.

To demonstrate the relevance of this finding in primary
tumors, we used a monoclonal anti-enolase-α antibody to analyze enolase-α expression status in NSCLC and its potential role in molecular classification of the tumors. We found that enolase-α is localized in both the cytoplasm and the nucleus in various cell types of normal lung tissue, including bronchial epithelium, a finding that is consistent with previous reports (8). However, 26% of the primary NSCLC exhibited substantial down-regulation of the molecule, and this down-regulation proportionally reduces staining in both the cytoplasm and the nucleus, supporting the notion that the proteins in the cytoplasm and nucleus are both enolase-α. Interestingly, tumors with enolase-α down-regulation tend to be larger than those without down-regulation, suggesting that the down-regulation is associated with tumor progression. The biological role of the down-regulation in NSCLC needs further investigation.

The association of the down-regulation of enolase-α with a poorer clinical outcome further supports the importance of this molecule in determining tumor aggressiveness in patients with NSCLC. It also indicates the importance of proteomic approaches in identifying potential biomarkers useful in cancer detection and classification. Our results should be validated in larger and independent studies to reach a definitive conclusion. It would also be interesting to determine whether up-regulation of enolase-α might have a therapeutic role in tumors lacking the molecule.

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