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

Yoon Soo Chang, Weiguo Wu, Garrett Walsh, et al.

Clin Cancer Res 2003;9:3641-3644.

Enolase-α Is Frequently Down-Regulated in Non-Small Cell Lung Cancer and Predicts Aggressive Biological Behavior[1]

Yoon Soo Chang, Weiguo Wu, Garrett Walsh, Waun Ki Hong, and Li Mao[2]

Molecular Biology Laboratory, Department of Thoracic/Head and Neck Medical Oncology [Y. S. C., W. W., W. K. H., L. M.] and Department of Thoracic and Cardiovascular Surgery [G. W.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas, 77030

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 and pathological stage, tumor histology, 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 ($P = 0.0398$).**

Conclusions: **Our results indicate down-regulation of enolase-α is common in NSCLC 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 NSCLC[3] (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 M_r 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 transformation of a transcriptional initiation complex (8, 9). MBP-1 mRNA is identical to enolase-α mRNA, which encodes a polypeptide of about M_r 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, M_r 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 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

Received 12/5/02; revised 4/14/03; accepted 4/17/03.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported in part by National Cancer Institute Grants CA 16620, CA 68437, CA86390, and CA91844; Tobacco Research Fund from the State of Texas; and Department of Defense Grant DAMD17-01-1-01689-1. W. K. H. is an American Cancer Society Clinical Research Professor.
[2] To whom requests for reprints should be addressed, at Molecular Biology Laboratory, Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 745 6363; Fax: (713) 796 8655: E-mail: lmao@mdanderson.org.

[3] The abbreviations used are: NSCLC, non-small cell lung cancer; MBP-1, *c-myc* binding protein-1; TBP, TATA-box binding protein; IHC, immunohistochemistry; CI, confidence interval.

Table 1 Analysis of the patients with NSCLC according to the expression status of enolase-α

	Normal expression	Down-Regulation	*P*
Age	64.3 ± 10.07	63.6 ± 13.24	0.394
Gender			
Male	18	6	
Female	16	6	1.000
Diameter of tumor	4.83 ± 2.72	8.32 ± 8.58	0.193
Tumor stage			
I, II	21	6	
III, IV	13	6	0.298
Tumor histology			
Adenocarcinoma	13	7	
Squamous carcinoma	15	4	
Large cell carcinoma	2	1	
Others	4	0	0.463
Differentiation			
Well differentiated	4	2	
Moderately differentiated	10	4	
Poorly differentiated	18	6	
Unclassified	2	0	0.911
Smoking status: *n* (%)			
Current; pack-year	17; 40.4 ± 22.98	2; 29.0 ± 25.52	0.183
Ex-smoker	12	7	
Nonsmoker	3	2	
Unknown	2	1	0.130
Median survival, mo (95% CI)			
Overall survival	43 (8.1–77.7)	6 (0–19.9)	0.040

razor blade and then were disrupted with a dunce homogenizer in ice-cold radioimmunoprecipitation buffer containing a protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). Phenylmethanesulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) stock solution was added, and the lysates were incubated on ice for 30 min, followed by centrifugation twice at 10,000 × *g* for 10 min at 4°C. The supernatant was taken and stored for protein analysis.

Western Blot Analysis. Twenty μg of protein extract were loaded into each well and were separated in 10% SDS-PAGE gel under reducing conditions. Separated proteins on the gel were electrotransferred onto Hybond-polyvinylidene difluoride membranes (Amersham Pharmacia Biotechnology, Arlington Heights, IL). Membranes were blocked with 5% nonfat dry milk to reduce background.

The membrane was incubated at 4°C overnight with anti-enolase-α monoclonal antibody (9C12) at a dilution of 1:50. This antibody, donated by Dr. E. F. Plow, does not cross-react with enolase-β and γ. β-actin was detected with a monoclonal anti-β-actin antibody AC-15 (Sigma) at a dilution of 1:1000. Then the blots were incubated with a sheep antimouse secondary antibody conjugated to horseradish peroxidase. Signals were detected by enhanced chemiluminescence on Hyperfilm ECL films (Amersham Pharmacia Biotechnology). Each signal was scanned and measured using the Scion Image program (Scion Corp., Frederick, MD). β-actin was used as an internal control. The level of enolase-α expression was calculated by dividing the signal of normal tissue by that of tumor tissue. Patient tissues were classified into two groups according to the level of enolase-α expression. One group included cases that did not show down-regulation of enolase-α (ratio, <2.0), and the other was composed of cases that did show down-regulation of enolase-α (ratio, ≥2.0).

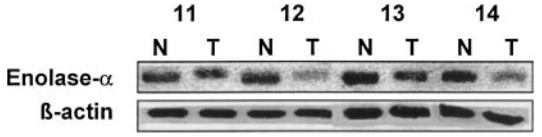

Fig. 1 Western blot of normal (*N*)-tumor (*T*) paired samples revealed that enolase-α is frequently down-regulated in primary NSCLC. β-actin was used as an internal control of protein loading.

Immunohistochemistry. IHC was performed in a conventional manner. Briefly, paraffin sections on slides were deparaffinized and rehydrated in serial graded ethanol. Antigen retrieval was skipped. Endogenous peroxidase activity was blocked in methanol containing 3% hydrogen peroxide. The blocking was performed by incubation in 5% horse serum, followed by overnight incubation with 1:100 anti-enolase-α antibody. After the sections were incubated with secondary antibody, the ABC complex and 3,3′-diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA) solutions were used serially, and the slides were counterstained with hematoxylin.

Statistical Analysis. In univariate analysis, independent sample *t* tests and χ^2 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. 2*A*). 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-

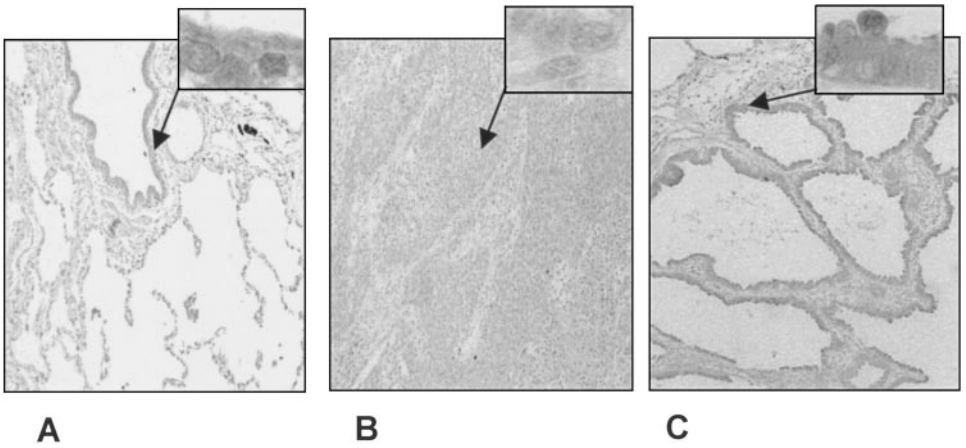

Fig. 2 IHC of normal lung tissue with enolase-α showed ubiquitous staining, including bronchial epithelium and alveolar structure (*A*). IHC of squamous lung cancer showed down-regulation of enolase-α that was consistent with the Western blot (*B*). Bronchioalveolar carcinoma, which generally has an excellent prognosis, showed a staining pattern similar to that of normal bronchial epithelium (*C*).

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).

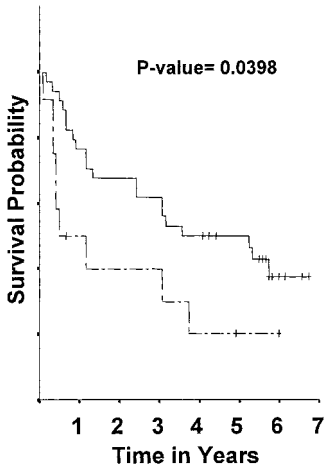

Fig. 3 Survival analysis demonstrated that patients whose tumors showed down-regulation of enolase-α had poorer overall survival compared with those whose tumors had normal expression levels of enolase-α.

DISCUSSION

Enolases have been characterized as highly conserved cytoplasmic glycolytic enzymes that catalyze the formation of phosphenolpyruvate 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 exhibits 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.

ACKNOWLEDGMENTS

We greatly appreciate the generosity of Dr. E. F. Plow at the Cleveland Clinic Foundation (Cleveland, OH), who provided the enolase-α monoclonal antibody (9G12) that was critical for this study. We also thank Georgia O. Lange in our tumor registry for providing clinical information.

REFERENCES

1. Ginsberg R. J., Vokes, E. E., and Rosenzweig, K. Non-small cell lung cancer. *In*: V. T. DeVita, Jr., S. Hellman, and S. A. Rosenberg (eds.), Cancer: Principles and Practice of Oncology, Ed. 6, pp. 925–983. Philadelphia-New York: Lippincott-Raven, 2001.

2. Weinberg, R. A. Oncogenes, anti-oncogenes, and the molecular basis of multistep carcinogenesis. Cancer Res., *49:* 3713–3721, 1989.

3. Marcu, K. B., Bossone, S. A., and Patel, A. J. *myc* function and regulation. Annu. Rev. Biochem., *61:* 809–860, 1992.

4. Potter, M., and Marcu, K. B. The *c-myc* story: where we've been, where we seem to be going. Curr. Topics. Microbiol. Immunol., *224:* 1–17, 1997.

5. Dalla-Favera, R., and Gaidano, G. Molecular pathogenesis of T-cell non-Hodgkin's lymphomas. *In*: V. T. DeVita, Jr., S. Hellman, and S. A. Rosenberg (eds.), Cancer, Principles and Practice of Oncology, Ed. 6, pp. 2228–2230. Philadelphia-New York: Lippincott-Raven, 2001.

6. Ray, R., and Miller, D. M. Cloning and characterization of a human *c-myc* promoter-binding protein. Mol. Cell. Biol., *11:* 2154–2161, 1991.

7. Ray, R. B. Induction of cell death in murine fibroblasts by a *c-myc* promoter binding protein. Cell Growth Differ., *6:* 1089–1096, 1995.

8. Ghosh, A. K., Steele, R., and Ray, R. B. Functional domain of *c-myc* promoter binding protein-1 involved in transcriptional repression and cell growth regulation. Mol. Cell. Biol., 2880–2886, 1999.

9. Chaudhary, D., and Miller, D. M. The *c-myc* promoter binding protein (MBP-1) and TBP bind simultaneously in the minor groove of the *c-myc* P2 promoter. Biochem., *34:* 3438–3445, 1995.

10. Subramanian, A., and Miller, D. M. Structural analysis of α-enolase: mapping the functional domains involved in down-regulation of the *c-myc* proto-oncogene. J. Biol. Chem., *275:* 5958–5965, 2000.

11. Feo, S., Arcuri, D., Piddini, E., Passotino, R., and Giallonogo, C. ENO-1 gene product binds to the *c-myc* promoter and acts as transcriptional repressor. FEBS Lett., *473:* 47–52, 2000.

12. Harris, R. A. Carbohydrate metabolism 1: major metabolic pathways and their control. *In:* T. M. Devlin (ed.), Textbook of Biochemistry with Clinical Correlations, Ed. 5, pp. 597–664. New York: Wiley-Liss, 2002.

13. Deloulme, J. C., Helies, A., Ledig, M., Lucas, M., and Sensenbrenner, M. A comparative study of the distribution of α- and γ–enolase subunit in cultured rat neural cells and fibroblasts. Int. J. Dev. Neurosci., *15:* 183–194, 1997.

14. Sensenbrenner, M., Lucas, M., and Deloume, J. C. Expression of two neuronal markers, growth-associated protein 43 and neuron-specific enolase, in rat glial cells. J. Mol. Med., *75:* 653–663, 1997.

15. Ida, H., and Yahara, I. Durable synthesis of high molecular weight heat shock proteins in G_0 cells of the yeast and other eucaryotes. J. Cell Biol., *99:* 199–207, 1984.

16. Sundrom, P., and Aliaga, G. R. Molecular cloning of cDNA and analysis of protein secondary structure of *Candida albicans* enolase, an abundant, immunodominant glycolytic enzyme. J. Bacteriol., *174:* 6789–6799, 1992.

17. Bisseret, F., Keith, G., Rihn, B., Amiri, I., Werneburg, B., Girardot, R., Baldacini, O., Green, G., Nguyen, V. K., and Monteil, H. *Clostridium difficile* toxin B: characterization and sequence of three peptides. J. Chromatogr., *490:* 91–100, 1989.

18. Johnstone, S. A., Waisman, D. M., and Rattner, J. B. Enolase is present at the centrosome of HeLa cells. Exp. Cell Res., *202:* 458–463, 1992.

19. Moscato, S., Pratesi, F., Sabbatini, A., Chimenti, D., Scavuzzo, M., Passatino, R., Bombardieri, S., Giallongo, A., and Migliorini, P. Surface expression of a glycolytic enzyme, α-enolase, recognized by autoantibodies in connective tissue disorders. Eur. J. Immunol., *30:* 3575–3584, 2000.

20. Redlitz, A., Fowler, B. J., Plow, E. F., and Miles, L. A. The role of an enolase-related molecule in plasminogen binding to cells. Eur. J. Biochem., *227:* 407–415, 1995.

21. Weith, A., Brodeur, G. M., Bruns, G. A., Matise, T. C., Mischke, D., Nizetic, D., Seldin, M. F., van Roy, N., and Vance, J. Report of the second international workshop on human chromosome 1 mapping 1995. Cytogenet. Cell Genet., *72:* 114–144, 1996.