

## Imaging, Diagnosis, Prognosis

# Identification of $\alpha$ -Enolase as an Autoantigen in Lung Cancer: Its Overexpression Is Associated with Clinical Outcomes

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**Abstract Purpose:** Although existence of humoral immunity has been previously shown in malignant pleural effusions, only a limited number of immunogenic tumor-associated antigens (TAA) have been identified and associated with lung cancer. In this study, we intended to identify more TAAs in pleural effusion – derived tumor cells.

**Experimental Design:** Using morphologically normal lung tissues as a control lysate in Western blotting analyses, 54 tumor samples were screened with autologous effusion antibodies. Biochemical purification and mass spectrometric identification of TAAs were done using established effusion tumor cell lines as antigen sources. We identified a p48 antigen as  $\alpha$ -enolase (ENO1). Semiquantitative immunohistochemistry was used to evaluate expression status of ENO1 in the tissue samples of 80 patients with non – small cell lung cancer (NSCLC) and then correlated with clinical variables.

**Results:** Using ENO1-specific antiserum, up-regulation of ENO1 expression in effusion tumor cells from 11 of 17 patients was clearly observed compared with human normal lung primary epithelial and non-cancer-associated effusion cells. Immunohistochemical studies consistently showed high level of ENO1 expression in all the tumors we have examined thus far. Log-rank and Cox's analyses of ENO1 expression status revealed that its expression level in primary tumors was a key factor contributing to overall- and progression-free survivals of patients ( $P < 0.05$ ). The same result was also obtained in the early stage of NSCLC patients, showing that tumors expressing relatively higher ENO1 level were tightly correlated with poorer survival outcomes.

**Conclusions:** Our data strongly support a prognostic role of ENO1 in determining tumor malignancy of patients with NSCLC.

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Identification of immunogenic tumor-associated molecules is not only a crucial step in understanding their roles on a molecular basis in cancer immunology, but also of interest for use in clinical diagnostic or therapeutic applications. Malignant pleural effusion is usually enriched with infiltrating lymphocytes and tumor cells. Therefore, it can be considered a local microenvironment for tumor cells. Tumor-associated immune complexes (1, 2) or autoantibodies (3, 4) have been found in effusion fluids, whereas only a limited number of immunoreactive targets were identified and associated with lung cancer (5, 6). Therefore, we collected malignant effusions from lung cancer patients to identify additional potential tumor-associated antigens using purified autologous effusion antibodies as probes. In the present study, we identified a p48 antigen as human  $\alpha$ -enolase (ENO1) through biochemical enrichment procedures and mass spectrometric analyses.

Enolase was originally characterized as an enzyme involved in glycolytic metabolism. More recent evidence, however, shows that enolase is a multifunctional protein. In mammalian cells, three isoforms of enolase have been found. They are designated as  $\alpha$ -(ENO1),  $\beta$ -(ENO3), and  $\gamma$ -(ENO2) enolases. The expression of these isoforms is developmentally regulated

in a tissue-specific manner. ENO1 is widely distributed in a variety of tissues, whereas ENO2 and ENO3 are exclusively found in neuron/neuroendocrine and muscle tissues, respectively (7). Enolases form heterodimers or homodimers to convert 2-phosphoglycerate into phosphoenolpyruvate in glycolysis. In addition to their glycolytic function, ENO1 was recently found to exist on the cell surface functioning as one of the plasminogen receptors (8), implying that it may play a role in tissue invasion. In hypoxic situations, it also acts as a stress protein that is up-regulated and speculated to provide protection to cells by increasing anaerobic metabolism (9). By using an alternative translation start codon, ENO1 transcripts can be translated into a MBP1-like p37, which is localized to nucleus and binds to the *c-myc* P2 promoter (10).

Development of high titers of autoantibody against enolases has been reported to be associated with a diverse range of systemic or organ-specific autoimmune diseases (11). In contrast, enolase-related autoantibody is rarely found in cancer patients in spite of the fact that another isoform,  $\gamma$ -enolase (neuron-specific enolase; ENO2), is a product of several types of tumors (12). One study identified ENO1 as antigenic target on human oral cancer cells that was recognized by autologous CD4<sup>+</sup> T cells (13). Immunoreactive sera to ENO1 were also recently found in 6.9% to 13.8% of patients with different subtypes of lung cancer (14, 15), suggesting that it can be an immunogenic target in cancer patients.

Changes in ENO1 gene regulation have been observed in several cancer cell models, whereas the clinical correlation of ENO1 expression to tumor states has not yet clearly been defined. Up-regulation of ENO1 has been reported in several highly tumorigenic or metastatic cell lines derived from either alveolar type II pneumocytes (16), small cell lung cancer (SCLC; ref. 17), or head and neck cancer (18). Similarly, studies examining enzymatic activities in breast cancer concluded a role for ENO1 in tumor progression (19). One recent bioinformatics study using gene chips and expressed sequence tag databases further supports a correlation between ENO1 expression and tumorigenicity as they found ubiquitously overexpression of ENO1 in 18 of 24 types of cancer, including lung cancer (20), strongly supporting its general pathophysiological role in cancer formation. However, clinical correlation between its expression status and tumor progression is quite controversial (21, 22) due to use of antibodies with different specificity for ENO1. Thus, we herein show specificity of our antiserum to  $\alpha$ -isoform by detection of known isoforms of human enolase using Western blot and immunoprecipitation analyses. Using this highly specific antiserum, we confirm that ENO1 is significantly overexpressed in effusion-derived tumor cells and tumor specimens of lung cancer. Coincidentally, its surface distribution is also detected and restricted in tumor cells only. Immunohistochemical analyses show that expression status of ENO1 in tumor is inversely correlated with prognoses of survival and disease recurrence for patients with non-SCLC (NSCLC). Therefore, ENO1 can be not only a potential target for targeting therapy, but also a potent prognostic marker for NSCLC patients.

## Materials and Methods

**Malignant pleural effusions and sample processing.** To identify immunogenic tumor antigens in lung cancer, 54 malignant pleural

effusion samples were collected. Histologic subtypes of the samples, depending on sample availability from our cooperated hospitals between 2001 and 2002 (under permission from Institutional Review Board, National Health Research Institutes, Taipei, Taiwan, Republic of China), include 4 small cell carcinoma, 45 adenocarcinoma, 5 squamous carcinoma, as well as 21 patients with non-cancer-associated diseases consisting of 13 pneumonia, 4 tuberculosis, and 4 heart disease. Separation of tumor cells from effusion-associated lymphocytes was done using a serial gradient centrifugation with Ficoll-Plaque Plus and Percoll (Pharmacia, Uppsala, Sweden) as described previously (23). The purity of tumor cells in fraction as determined by cytologic examinations was between 70% and 90%.

**Effusion antibody preparation.** The immunoglobulins of pleural effusions were partially purified using ammonium sulfate precipitation. One volume of effusion fluid was added dropwise with 0.66 volume of saturated ammonium sulfate with constant mixing for 1 hour followed by centrifugation at 10,000  $\times$  g at 4°C for 10 minutes. The resultant pellet was dissolved in 0.1 volume of distilled water and dialyzed against PBS overnight.

**Cells and flow cytometry analysis.** The primary effusion tumor cells were obtained from a 51-year-old patient (CA926) with poorly differentiated lung adenocarcinoma and grown in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1 mmol/L sodium pyruvate (Invitrogen, Grand Island, NY), 0.1 mmol/L nonessential amino acid (Sigma-Aldrich, St. Louis, MO), 2 mmol/L glutamine, 50  $\mu$ g/mL streptomycin, and 500 units/mL penicillin for two passages. The NHRI-L89 (abbreviated as L89) cell line was originally obtained from effusion tumor cells of a 36-year-old patient with stage IV lung adenocarcinoma and characterized as cytokeratin (+)/calretinin (-). The cells have been cultured in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mmol/L glutamine, and antibiotics for at least 40 passages *in vitro*. Normal bronchial/tracheal and small airway primary epithelial cells were purchased from Cambrex (Walkersville, MD) and cultured in medium provided by the manufacturer.

For flow cytometry analysis, cells isolated from cancer-associated or non-cancer-associated effusion fluids were cultured in ACL-4 (24) and RPMI 1640 (1:1) for one or two passages. The intact whole cells were stained with or without ENO1 antibody, visualized with goat antiserum-conjugated Cy2 (Jackson ImmunoResearch Laboratories, West Grove, PA), and analyzed using the FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

**Protein purification and identification.** The p48 antigen (ENO1) was purified using DEAE anionic column, ammonium sulfate fractionation, and long-range SDS-PAGE separation. The L89 cells were lysed with cell lysis buffer [20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP40] containing protease inhibitor cocktail (Calbiochem, La Jolla, CA). Approximately 45 mg of the detergent-soluble fraction were fractionated in a HiTrap DEAE column (Amersham Biosciences, Uppsala, Sweden) buffered with 20 mmol/L Tris-HCl (pH 7.0) and eluted with increasing concentration of NaCl ranged from 0 to 1.0 mol/L at 1 mL/min pressure. To determine the abundance of p48 antigen in each fraction, the elutants were dialyzed against PBS and detected by Western blot analysis using the CA926 effusion antibody as a probe. Subsequently, the p48-enriched fraction (~2.5 mg) was subjected to ammonium sulfate fractionation (25). Each dialyzed fraction (50  $\mu$ g) was resolved on 10% SDS-PAGE in duplicate and visualized by quick silver staining (26) or Western blotting. The putative p48 band on the silver-stained gel was excised and analyzed by Babraham Bioscience Technologies, Inc. (Cambridge, United Kingdom) using an application programming interface Q-Star ESI-Quadrupole-TOF.

**Gene cloning and antiserum generation.** To generate recombinant ENO1 protein, the gene was cloned out from L89 cells by reverse transcription-PCR using gene-specific primers 5'-GGTGAATTCATC-TATTCTCAAGATCCATGCC-3' (forward) and 5'-ACTCCATGGT-TACTTGGCCCAAGGGTTTCT-3' (reverse). The resultant PCR fragment was digested with *Eco*RI and *Nco*I, cloned into pGEX-KG vector, and expressed in *Escherichia coli* cells to generate a recombinant protein

**Table 1.** Correlation of expression status of ENO1 and patient-related clinical variables using Quick score of 5 as a cutoff value

Variable	ENO1 expression				
	Total, n (%)	Score <5, n (%)	Score ≥5, n (%)	P (univariate)	P (multivariate)
Quick score	80 (100)	17 (21)	63 (79)	1.0	0.926
Medium age (y)					
<65	29 (36)	6 (21)	23 (79)	1.0	0.926
≥65	51 (64)	11 (22)	40 (78)		
Gender					
Male	69 (86)	15 (22)	54 (78)	1.0	0.789
Female	11 (14)	2 (18)	9 (82)		
Smoking status					
No	25 (31)	8 (37)	17 (68)	1.0	0.665
Yes	55 (69)	15 (27)	40 (73)		
Histology					
SCC	40 (50)	8 (20)	32 (80)	1.0	0.785
Non-SCC	40 (50)	9 (22)	31 (78)		
AdenoCA	31 (39)	6 (19)	25 (81)	0.787	0.742
Non-adenoCA	49 (61)	11 (22)	38 (78)		
Stage					
I + II	51 (64)	15 (29)	36 (71)	0.022*	0.018*
III	29 (36)	2 (7)	27 (93)		
Recurrence					
No	35 (44)	12 (34)	23 (66)	0.015*	0.012*
Yes	45 (56)	5 (11)	40 (89)		

NOTE: Univariate analysis was done using Fisher's exact test. Multivariate analysis was done using logistic regression method.

Abbreviations: SCC, squamous cell carcinoma; adenoCA, adenocarcinoma.

\*P < 0.05, indicates statistically significant.

fused with glutathione S-transferase (GST) tag. Protein purification was done using glutathione-immobilized affinity chromatography as recommended by a manufacturer (Sigma-Aldrich). ENO1 rabbit antiserum raised by immunizing with the recombinant protein was produced by Kelowna, Inc. (Taipei, Taiwan, Republic of China). To avoid cross-reaction with the tag protein, the serum was further absorbed with immobilized GST resins (Pierce, Rockford, IL).

To determine the isoform-specific recognition of CA926 antibodies and the ENO1-specific rabbit antiserum, human ENO2 and ENO3 genes were cloned out from CA926 tumor cDNA and human heart (Stratagene, La Jolla, CA), respectively, by reverse transcription-PCR. The isoform-specific primers used for the gene cloning are listed as follows: ENO2, 5'-ATTGAATTCTTCATAGAGAAGATCTGGGCCCGGGAGAT-3' (forward) and 5'-ATTGAATTCTCACAGCACACTGGGATTACGGAAG-3' (reverse); ENO3, 5'-AGGGAATTCTGCCATGCAGAAAATCTTTGC-3' (forward) and 5'-ATTGAATCTCAGTGGCCTTCGGGTT-3' (reverse). The resultant fragments were digested with EcoRI, cloned into pBlueScript-myc vector, and overexpressed in HeLa cells by infection of T7 vaccinia viruses as described previously (27). Construction of the human ENO1 gene in pBlueScript-myc was done in the same manner.

**Immunoblotting.** To detect p48, ENO-1, ENO-2, ENO-3, or Myc-tagged proteins, 50 µg of cell lysates were resolved by 10% SDS-PAGE using appropriate antibodies in Western blotting analyses, which were described in each figure legends. The immunocomplex was visualized by SuperSignal chemiluminescence (Pierce). Glyceraldehyde-3-phosphate dehydrogenase and β-actin are loading controls. The ENO2- and ENO3-specific monoclonal antibodies were obtained from Abnova Co. (Taipei, Taiwan, Republic of China).<sup>14</sup> Myc-tag (9E10), glyceraldehyde-3-phosphate dehydrogenase, and β-actin were purchased from Upstate Biotechnology (Lake Placid, NY), Biogenesis (Poole, United Kingdom), and Sigma Co. (St. Louis, MO), respectively.

**Patients.** The acquisition of paraffin tissues was approved by local Institutional Review Boards. Formalin-fixed, paraffin-embedded tissue samples were obtained from 80 patients with NSCLC, including 40 squamous cell carcinoma, 31 adenocarcinoma, 4 adenosquamous cell carcinoma, and 5 large cell carcinoma. The histology of tumor types was determined according to WHO classification, and disease stages were scored by tumor size and node metastasis. No patients received either adjuvant or neoadjuvant therapy. Surgery was done from January 2000 to December 2001 with complete follow-up at Taichung Veterans General Hospital (Taichung, Taiwan, Republic of China). Clinical data of the patients are summarized in Table 1. Progression-free survival (PFS) was calculated from the date of surgery to the date of disease recurrence or death. Data for patients who were alive and relapse-free were censored as of the date of the last following-up visit. Overall survival (OS) was counted from the date of surgery to the date of death. Eighty patients were followed for up to 60 months after surgery. The median follow-up duration of the patients was 29.5 months. Among these 80 patients, 30 patients died as a result of lung cancer and had a median OS of 10 months ranging from 2 to 42 months. Forty-five patients experienced disease recurrence.

**Immunohistochemistry.** Immunohistochemistry was done using a conventional method. The sections were rehydrated, washed with PBS, and followed by incubation with the ENO1 antiserum (1:2,000). The specificity of the antiserum was also examined by staining sections of the same tissue block with preimmunized serum, antiserum against GST tag, or ENO1 antiserum preabsorbed with 1 mg of membrane-immobilized ENO1 antigen in 1:2,000 dilution. After the sections were incubated with biotinylated secondary antibody, the avidin-biotin complex method was visualized by exposing sections to 3,3'-diaminobenzidine solution (DAKO, Carpinteria, CA) and the slides were counterstained with hematoxylin.

**Quantification of ENO1 expression and statistical analysis.** The expression status of ENO1 was assessed semiquantitatively using Quick score method (28) at a final magnification of ×200 independently by two pathologists in a blinded manner. Conflicting scores were

<sup>14</sup> <http://www.abnova.com.tw>.

resolved at a discussion microscope. The Quick score method is a semiquantified approach based on intensity and heterogeneity of immunohistochemical staining and has been shown to give the same statistically significant correlations with clinical outcomes of breast cancer patients when compared with conventional scoring termed "Category" system (28). The intensity of staining was scored as 0, 1, 2, and 3 standing for negative, weak, moderate, and strong staining, respectively. For the heterogeneity staining, the proportion of tumor cells staining positively was scored as follows: 0, 0%; 1, 1% to 25%; 2, 26% to 50%; 3, 51% to 75%; 4, 76% to 100%, compared with the total of tumor cells. Scores obtained from intensity and heterogeneity of positivity were added to give a final Quick score ranged from 0 to 7. The numbers of patients with respect to the intensity and distribution of ENO1 staining are shown in Supplementary Table S1 file.

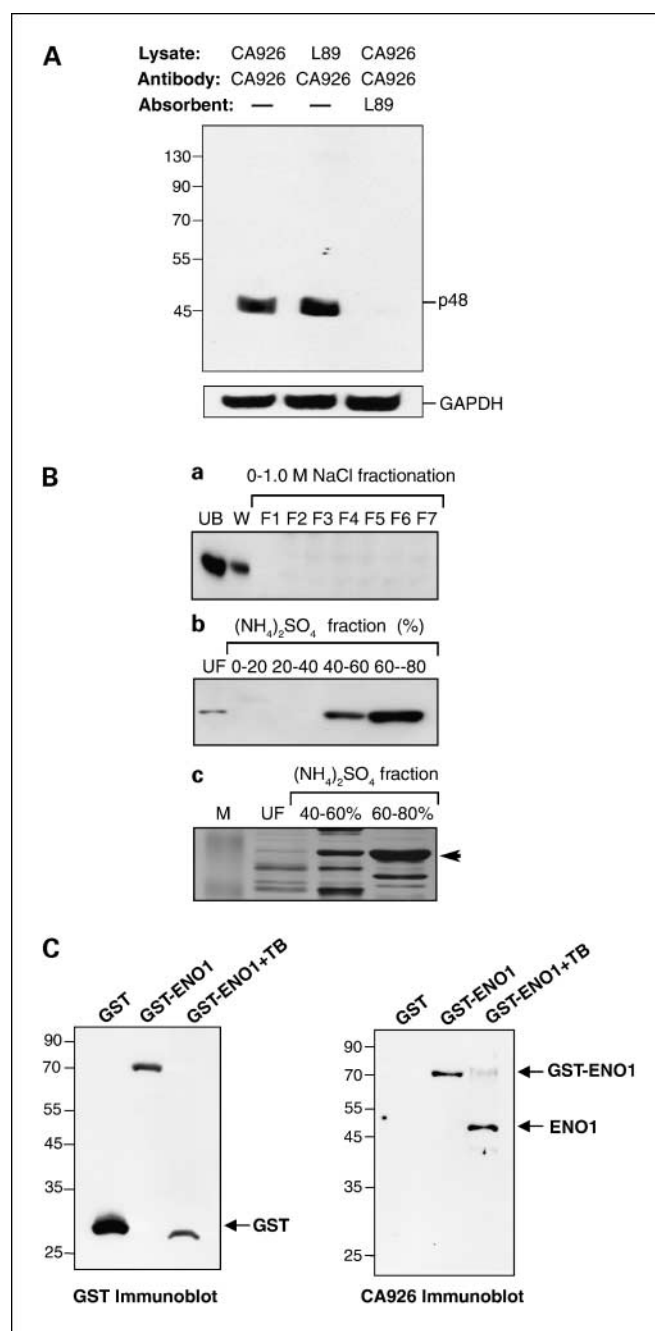
Because a large number of tumors highly expressed ENO1, the score for the immunohistochemical staining all shows  $\geq 3$ . None of the score gave a fair separation between patients with different clinical variables shown in Table 1. We tested a cutoff value for Quick score system starting from the score 3 to 7. Because of a limited number of 80 patients in the score of  $<4$  or  $\geq 7$ , reasonable scores are the score of 5 or 6. Using the score of 6 as a cutoff value, none of clinical variables were significantly associated with ENO1 expression status. Therefore, the score of 5 as a cutoff value was chosen for continuing following analyses and to define higher or lower expression of ENO1 in tumors.

The associations between ENO1 expression status (score  $<5$  or  $\geq 5$ ) and clinical variables were calculated with univariate (Fisher's exact test) and multivariate (logistic regression) methods. Survival curves for PFS and OS were plotted according to Kaplan-Meier method, and significances of differences between groups were analyzed by a log-rank test. A Cox multivariate analysis was used to further explore observed differences and identify factor(s) independently contributing to survivals of cancer patients. The factors included in this analysis were the expression status of ENO1, age, gender, smoking status, histologic types, tumor stages, and recurrence status shown in Table 1.  $P < 0.05$  is considered to be statistically significant.

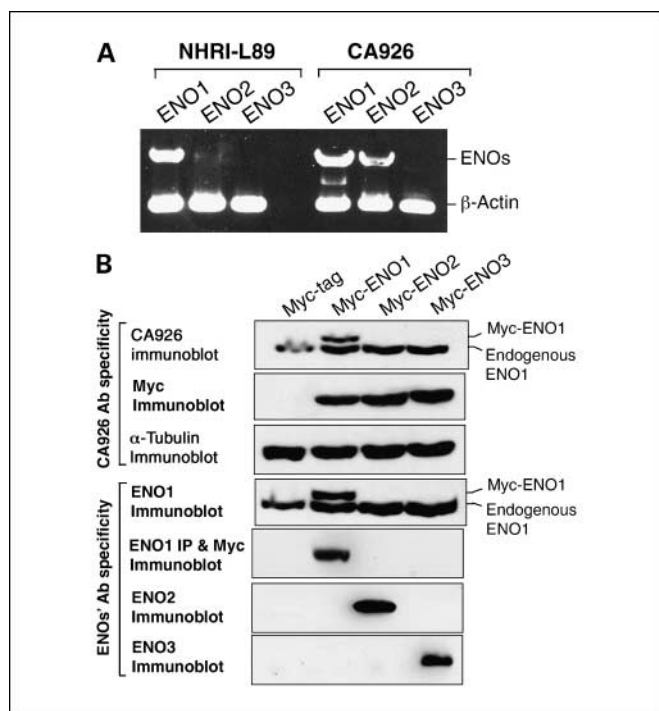
## Results

**p48 antigen recognized by CA926 autologous effusion antibodies.** To detect existing humoral immunity in pleural effusions, we examined 54 lung cancer patients by Western blot analyses of tumor cell-enriched fractions with purified autologous effusion antibodies. Enrichment of effusion-associated tumor cells was carried out by serial gradient centrifugations with Ficoll and Percoll as described previously (23). Subsequently, using morphologically normal lung tissues as a control lysate in Western blotting analyses, we have detected 4 of 54 patients with antibodies recognizing one or two proteins overexpressed or uniquely expressed in the autologous tumor cells. However, none of distinct proteins were detected in 21 patients with non-tumor-associated diseases in this screening (data not shown). Patient CA926 is one of the four patients whose effusion antibodies specifically recognize a 48-kDa (p48) major protein in autologous tumor cells. The same antigen also appeared in another effusion cancer cell line, L89. The antigen identity was confirmed by a competition experiment using CA926 effusion antibodies preabsorbed with the L89 lysate (Fig. 1A). Therefore, this cell line was used as the source for the protein identification in this study.

**Purification and identification of p48 antigen.** Identification of the p48 antigen was done by biochemical purification and mass spectrometry analysis (Fig. 1B; Supplementary Fig. S1). Enrichment of the p48 antigen was carried out by DEAE chromatography purification, ammonium sulfate "salting-out"



**Fig. 1.** Purification and identification of immunogenic p48 antigen as human ENO1. **A**, top, p48-enriched lysate (30  $\mu\text{g}$ ) from each cells was electrophorized and immunoblotted with CA926 antibodies or the antibodies preabsorbed with immobilized L89 lysate. The immunoreactions were carried out in an incubation manifold (Hoefer, San Francisco, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein loading control. Left, molecular weight markers in kDa. **B**, detergent-soluble L89 lysate was dialyzed and precleared by centrifugation before purification. **a**, lysate was loaded onto a DEAE column. The bounded proteins were fractionated by increasing concentration of NaCl. Western blotting of unbound (UB), washout (W) lysates, and each fractionated elutants (F1-F7) was done and probed with CA926 effusion antibodies. **b**, unbound p48-enriched lysates were further fractionated with saturated ammonium sulfate, ranged from 0% to 80%, and immunodetected with CA926 antibodies. **c**, ammonium sulfate-fractionated lysates were resolved on 10% SDS-PAGE in duplicate. One was probed with CA926 antibodies. The other was visualized by silver staining. The putative immunoreactive protein band in the gel was excised for mass spectrometry analysis. **C**, gene encoding for human  $\alpha$ -enolase (ENO1) was cloned out from L89 cells and expressed as a GST fusion protein. Bottom, Western blotting of 1  $\mu\text{g}$  of GST tag protein and ENO1 recombinant protein with or without thrombin (TB) treatment was hybridized with either GST or CA926 antibodies.



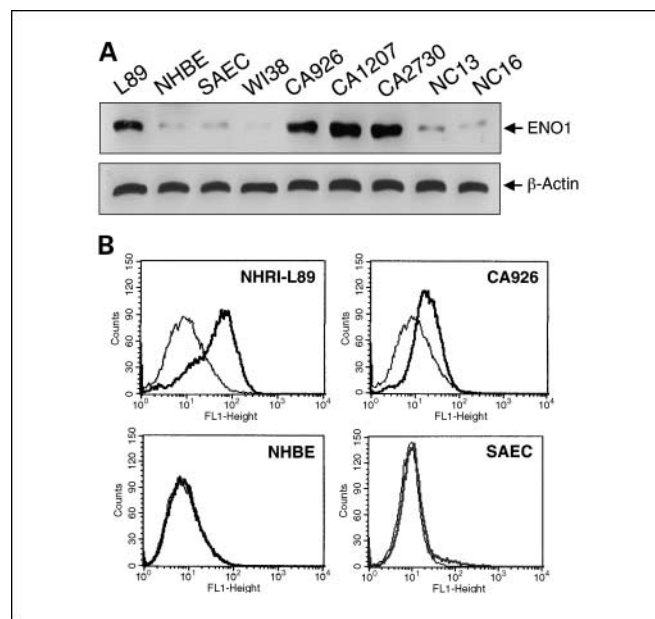
**Fig. 2.** Specificities of CA926 and rabbit ENO1 antibodies (*Ab*) to enolase isoforms. *A*, total RNA was extracted from L89 or CA926 tumor cells and reverse transcribed into cDNA pools. Abundance of each isoform gene was determined by reverse transcription-PCR analysis using a pair of isoform-specific primer as listed in Materials and Methods.  $\beta$ -Actin gene was used as an internal control. *B*, top, HeLa cells were transfected with a plasmid expressing Myc-tagged ENO1, ENO2, ENO3, or empty vector (Myc-tag). Left, Western blot of each transfected lysates was resolved on a 10% SDS-PAGE and probed with antibodies. For immunoprecipitation (*IP*) experiment, each transfected HeLa lysate was hybridized with ENO1 antiserum, precipitated with protein G beads, and probed for Myc-tagged proteins. Right, endogenous ENO1 in HeLa cells was labeled.

precipitation, and SDS-PAGE separation. The DEAE column buffered with 20 mmol/L Tris-HCl (pH 7.0) optimally bound 94.6% of loaded proteins but excluded the p48 from the column. The flow-through and wash fractions containing p48 subsequently were fractionated with increasing saturations of ice-cold ammonium sulfate. The result indicates that the antigen was concentrated primarily in a 60% to 80% fraction. The enriched fraction was then electrophorized and visualized by silver staining. The corresponding immunoreactive protein band was excised for mass spectrometry analysis. As data shown in Supplementary Fig. S1, six trypsinized peptides were sequenced and the p48 antigen was identified as human  $\alpha$ -enolase (ENO1; 2-phospho-D-glycerate hydrolase). To confirm ENO1 as the target of CA926 antibodies, the gene encoding ENO1 was cloned out from the L89 cells and fused with GST tag sequence for protein purification. The tag protein was then removed by thrombin enzymatic digestion. Western blot in Fig. 1C clearly shows that CA926 antibodies specifically recognize ENO1 and its fusion proteins but not the GST, confirming that ENO1 is the major immunogenic target of CA926 effusion antibodies in the tumor cells.

**Isiform-specific recognition of CA926 antibodies.** Because the three mammalian cloned isoforms of enolase share ~84% protein sequence identity, we first checked for the presence of these isoforms in CA926 and L89 cells. Using isoform-specific primers, reverse transcription-PCR analyses showed that CA926

cells expressed both ENO1 and ENO2 but not ENO3, whereas only ENO1 was detected in L89 cells (Fig. 2A). To investigate the specificity of CA926 effusion antibodies, the genes encoding ENO2 and ENO3 were cloned out from CA926 and human heart cDNA pools, respectively, and expressed in HeLa cells. As shown in Fig. 2B, CA926 antibodies consistently exhibited high specificity to ENO1 but failed to recognize ENO2 and ENO3. However, when further detecting titers of ENO1-specific effusion antibodies in those patients, Western blotting analysis surprisingly showed only 7.4% of the cancer patients, including patient CA926, versus 52.3% of the non-cancer patients to have the antibody (data not shown). ELISA analyses using the purified recombinant ENO1 as a target consistently showed significantly lower titers of anti-ENO1 antibodies found in the cancer patients compared with those in patients with non-cancer-associated diseases and healthy subjects (Supplementary Fig. S2). The concentrations of total immunoglobulins (IgA, IgG, and IgM) were still unchanged, suggesting that ENO1-mediated immune modulation probably occurs in the majority of the lung cancer patients.

**Overexpression of ENO1 in lung tumor cells and tissues.** To test whether ENO1 is abnormally expressed in tumor cells, two types of human lung primary cells, normal human bronchial epithelial and small airway epithelial cells, were used for controls. We raised rabbit antiserum against recombinant ENO1 protein and examined its specificity to individual isoforms of enolase by Western blotting and immunoprecipitation analyses. As shown in Fig. 2B, the antiserum clearly shows ENO1-restricted recognition. To determine ENO1



**Fig. 3.** Overexpression and surface staining of ENO1 in effusion tumor cells. *A*, tumor or mesothelial cells were obtained from pleural effusions of 17 patients with lung cancer or 6 patients with non-cancer-associated diseases, respectively. Among them, tumor cells from four cancer patients, including L89 cells, and effusion cells from two control patients are representative cases. Normal human bronchial epithelial (*NHBE*) and small airway epithelial (*SAEC*) cells are normal human lung primary cells. WI38 is lung embryonic fibroblast cell line. Cell lysates (30  $\mu$ g) were resolved on 10% SDS-PAGE and immunoblotted with ENO1 antiserum. After stripping, the same blot was reprobed with  $\beta$ -actin antibody as a loading control. *B*, intact whole cells were stained with (*dark line*) or without (*gray line*) ENO1 antiserum. L89, CA926, and two lung primary control cells are representative cases.

expression level in effusion cells, 17 of 54 patients with lung cancer and 6 of 21 patients with non-cancer-associated diseases containing sufficient amount of effusion cells were examined. CA926, L89, and 11 of 17 patients have at least 2-fold increase in ENO1 expression when compared with its level in normal human bronchial epithelial, small airway epithelial, and effusion cells from the 6 control patients (Fig. 3A). Intriguingly, these overexpressed tumor cells all stained positive for cell surface ENO1 as well (Fig. 3B), supporting previous studies (8, 29) that surface ENO1 may have a role in promotion of plasmin generation or cell invasion.

To determine the expression of ENO1 in lung tumors, immunohistochemical analyses were done on 80 samples. The tumor parts in all samples manifested clear evidence of ENO1 immunoreactivity. More than 25% of total tumor cells were stained positively in 76 of 80 samples. Dominant cytoplasmic staining was observed in all tumor sections (Fig. 4A, C, and D); additionally, distinct membranous or nuclear staining was also detected in some tumor cells. In contrast, stromal tissues adjacent to tumors and normal-like alveolar epithelial cells distal to tumors showed low basal staining (Fig. 4A and C). Notably, alveolar cells proximal to the tumor displayed increasing immunoreactivity in either the cytoplasmic, nuclear, or membranous regions (Fig. 4C, *inset*, *arrows*). Another two negative controls using ENO1 antiserum preabsorbed with immobilized purified ENO1 antigen (Fig. 4B) and antiserum against GST protein (data not shown) were also included in this study. Data taken from Western blot and immunohistochemical analyses strongly indicate that increased ENO1 expression is prevalent in patients with NSCLC.

**Association of ENO1 expression with clinical outcomes.** To evaluate the relevance of ENO1 expression with clinical outcomes of the patients, its expression status was quantified by Quick score method (28). When a cutoff value of score 5 was used, expression status of ENO1 in tumor tissue was significantly correlated with tumor stages ( $P = 0.018$ ) and disease recurrence ( $P = 0.012$ ) shown in Table 1. Tumors in patients with stage III disease (93%) or disease recurrence (89%) had higher ENO1 overexpression (score  $\geq 5$ ) compared with patients with stage I/II disease (71%) or no detected recurrence (66%). No statistical differences in age, gender, smoking status, or histologic subtypes were found between patients with lower ( $<5$ ) or higher ( $\geq 5$ ) ENO1 expression.

Survival analysis according to Kaplan-Meier method revealed significant correlation between ENO1 expression status and either PFS or OS in all patients (Fig. 5A and B). With log-rank test, we first tested association between patients with stage I/II and III disease (Supplementary Fig. S3). As expected, patients with stage III disease had much poorer PFS and OS than patients with stage I/II disease. Similarly, in Fig. 5A and B, patients with tumors expressing higher levels of ENO1 (score  $\geq 5$ ) also bore a tight correlation with poorer PFS and OS significantly ( $P = 0.0099$  and  $0.0027$ , respectively). Moreover, to avoid a bias favoring higher ENO1 expression as a poor prognosis marker due to a large number of stage III tumors with the score  $>5$ , patients with stage I/II disease were included for analysis. Results in Fig. 5C and D consistently showed that its expression level was reversely correlated with PFS and OS significantly ( $P = 0.04$  and  $0.034$ , respectively). Moreover, when even using the score of 6 as a cutoff value, log-rank survival analyses also show that stage I/II patients with tumors

expressing ENO1 level  $<6$  have a good prognosis for PFS ( $P < 0.001$ ) and a better trend for OS ( $P = 0.18$ ), although the score of 6 as a cutoff value for ENO1 expression was shown no significant association with the clinical variables (Supplementary Table S2). Thus, these data strongly support a potential prognostic role of ENO1 expression for NSCLC patients.

Cox multivariate analysis was used to test contribution of ENO1 expression status, tumor stage and/or disease recurrence, age, gender, smoking status, and histologic subtypes on PFS and OS of the patients. Only ENO1 expression with score  $\geq 5$  proved to be associated with poor PFS ( $P = 0.031$ ). In contrast, stage I/II disease was shown to have a better trend on PFS ( $P = 0.076$ ). Additionally, both lower ENO1 expression (score  $<5$ ) as well as free of disease recurrence were emerged as strong factors contributing on better OS outcomes ( $P = 0.008$  and  $0.003$ , respectively). Taken together, these data strongly support expression status of ENO1 to be a potent prognostic marker for survival outcomes of NSCLC patients.

## Discussion

Because molecular identity of tumor-associated antigens is first discovered in melanoma patients, this finding has led to elucidation of tumor immunity, including cellular and humoral immunities, at a cellular and molecular level. Interestingly, the majority of these antigens characterized to date are unaltered self-antigens. In the present study, we also identified a 48-kDa protein of CA926 tumor cells as unaltered human  $\alpha$ -enolase (ENO1), which is recognized by autologous effusion antibodies. As predicted by a previous bioinformative study (20), ENO1 is overexpressed in lung cancer in our study. About 65% of patients with effusion tumor cells and all the tumor samples we have examined thus far consistently show up-regulation of ENO1 gene expression in the tumors compared with that in normal primary lung epithelial cells and tumor-adjacent normal tissue counterparts. Moreover, the statistical analyses correlating ENO1 expression in the patients with their clinical outcomes further reveal that the expression status of ENO1 is highly associated with survival and disease recurrence of NSCLC patients.

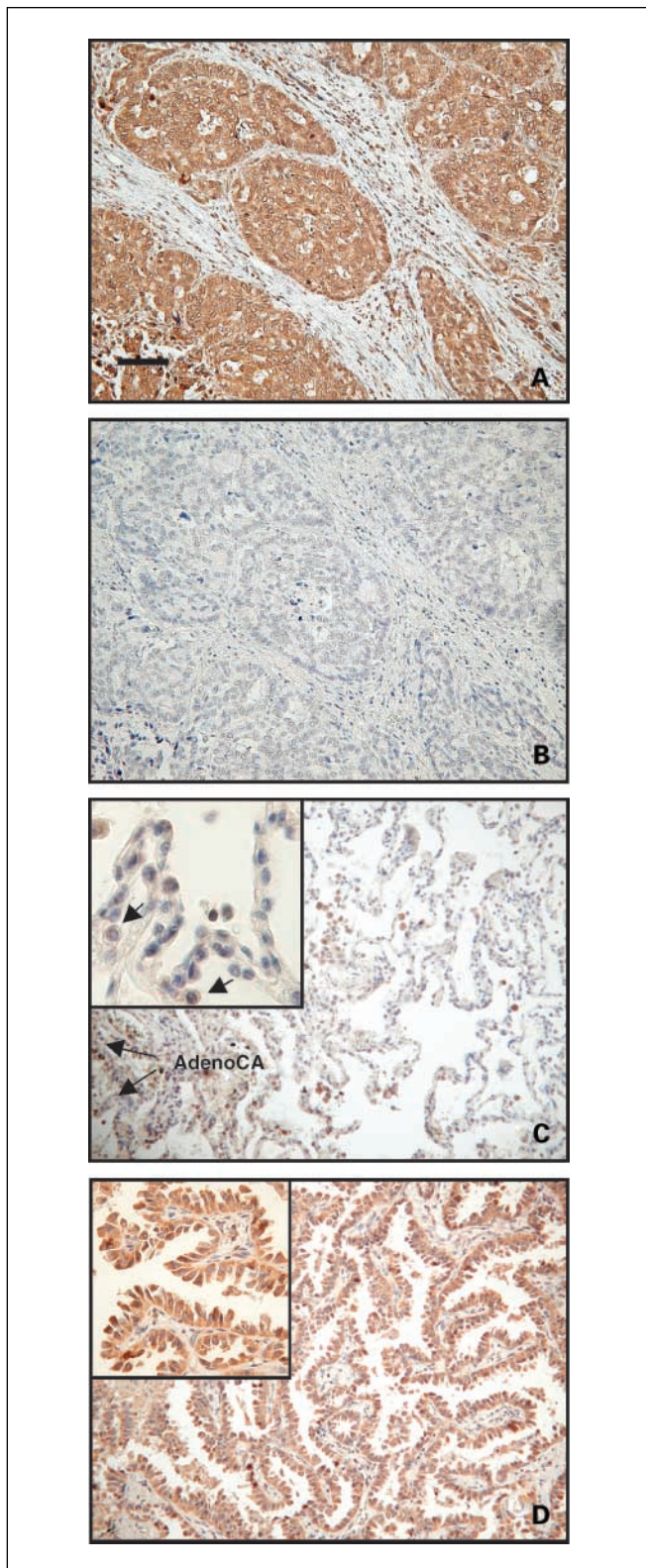
Overexpression of unaltered antigen(s) in malignant cells is believed to be one of possible mechanisms to initial tumor immunity (30), whereas the immunity is eventually suppressed resulting in tumor escape. B7-H1 (31), IDO (32), RCAS1 (33), etc. have been shown to overexpress in a variety of cancers, including lung cancer. However, autoantibodies immunoreactive to these antigens are rarely reported in cancer patients but associated with some inflammatory or metabolic diseases (34, 35), implicating that tumor antigen-mediated immunosuppression may occur in tumor malignancy. Consistent with previous studies to detect CML28 (36) and ENO1 (14) autoantibodies in chronic myelogenous leukemia and lung patients, respectively, our data obtained from Western blotting analyses also show that only a small percentage (7.4%) of the cancer patients, including patient CA926, develop high titer of antibodies against ENO1. The same conclusion is also observed when a sandwich ELISA to quantify levels of the autoantibodies was used, showing that the levels in the cancer patients are significantly lower ( $P < 0.01$ ) than those in effusion fluids of the non-tumor-associated patients and in sera of healthy control subjects (Supplementary Fig. S2).

Failure to exert antitumor effect may be associated with defects in complement-mediated cytotoxicity. Because antibody-induced complement attack is one of main themes for tumor cell lysis, quantitative requirement of C3 to activate downstream membrane attack complex is indispensable (37).

Thus, we further detected levels of C3 and C4 in patient CA926. The values are 29.2 mg/dL for C3 and 4.7 mg/dL for C4, which all are far below reference ranges (C3, 90-180 mg/dL; C4, 10-40 mg/dL). This preliminary data suggest that lack of sufficient amount of C3 and C4 may account for, at least partially, tumor escape in patient CA926. Intriguingly, a recent study showed that treatment of mice with the recombinant bacterial enolase could stimulate production of interleukin-10, a pro-Th2 cytokine (38). Therefore, it would be interesting to further investigate whether overexpression of ENO1 in malignant cells is involved in Th1 and Th2 switch because active CD4<sup>+</sup> T help cells have indispensable role in development and maintenance of CTLs and antibody-secreting B cells.

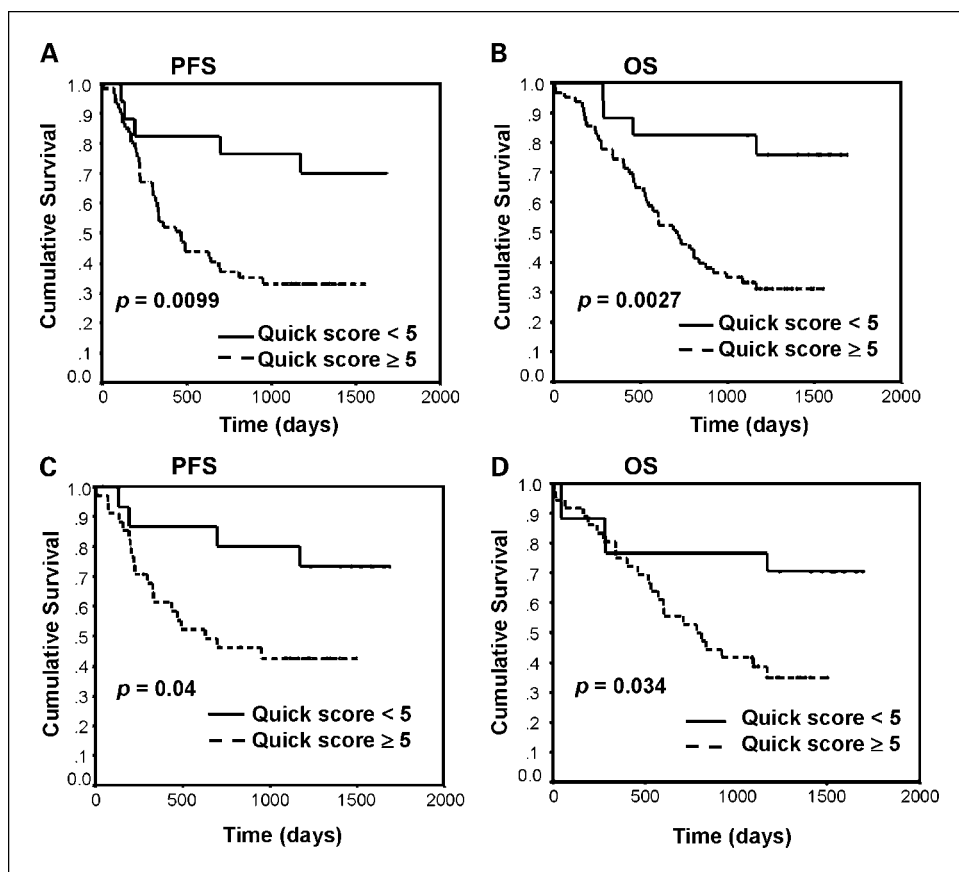
Up-regulation of glycolytic enzymes is a common scenario in a variety of cancers. In response to hypoxia, normal cells will increase gene expression of glycolytic enzymes to adapt environmental stress through activation of hypoxic-inducible transcription factor (39). However, this cellular process is reversible for normal cells but irreversible for malignant cells. Moreover, tumor cells preferentially use anaerobic glycolysis for inefficient energy metabolism (Warburg effect), leading to increase difficulties for disease treatment. More recently, oncogenic AKT and Myc have been shown to stimulate anaerobic glycolysis directly and ENO1 is one of the Myc-driven target genes (40). Thus, elevated level of ENO1 gene expression seems to be an inevitable consequence during tumorigenesis. Consistent with previous reports on cell line studies, we further show that ENO1 is overexpressed in patients with lung cancer and its high levels (score  $\geq 5$ ) are tightly correlated with poor prognoses.

However, a previous study (21) obtaining a conclusion different from ours may be attributed to different isoform-specific antibodies used in the experiments. Because the three known isoforms of mammalian enolases are highly conserved in protein sequence (~84% protein identity), it would be a difficult task to generate isoform-specific antibody for each of them. We raised antisera against purified recombinant ENO1 protein from three rabbits. Only antiserum from one rabbit displayed high specificity to ENO1. To confirm its recognition specificity, we cloned out all the isoforms and showed its specificity in three ways: (a) immunoreactivity of the antibody specific to ENO1 but not to human ENO2 and ENO3 in Western blotting analyses (Fig. 2B), (b) capability and specificity of the antibody to immunoprecipitate ENO1, and (c) abolishment of immunohistochemical staining by absorption of the antibody with the purified recombinant ENO1 (Fig. 4). Using the antibody, we exhibit relatively lower levels of ENO1 in two human primary lung epithelial cell controls and normal lung tissues compared with those in effusion tumor cells and tumor tissues. These data corroborate Holland et al. (41) initial observations that showed an increased expression



**Fig. 4.** Immunohistochemical staining of ENO1 in lung tumor tissues. Tissue sections from patients with lung cancer were examined immunohistochemically. Transverse serial sections were obtained from a patient with lung adenocarcinoma (*AdenoCA*) and stained with ENO1 antiserum (*A*) or ENO1 antiserum preabsorbed with immobilized GST-fused ENO1 antigen (*B*) to show specificity of ENO1 antiserum. Normal-like alveolar epithelial cells proximal to the tumor part (*arrows*) displayed increasing staining of ENO1 in either cytoplasm, nuclear, or membrane (*inset, arrows*), whereas the distal alveolar walls (*C*) showed low basal expression. *D*, inset, adenocarcinoma cells in another patient also showed high level of cytoplasm staining of ENO1. Magnifications,  $\times 100$  (*A-D*) and  $\times 200$  (*C* and *D*, *insets*). Bar, 100  $\mu\text{m}$ .

**Fig. 5.** Kaplan-Meier analyses of PFS and OS. PFS in (A) and OS in (B) were expressed as a function of Quick score  $<5$  or  $\geq 5$  for ENO1 expression levels in 80 patients. Fifty-one patients with stage I/II disease were analyzed for correlation between ENO1 expression status ( $<5$  or  $\geq 5$ ) and survivals [PFS (C) or OS (D)]. Bottom left,  $P$ -values.



of ENO1 in exponentially growing cells, whereas expression in resting phase cells was at very low levels. In contrast to our antiserum, because the specificity of 9G12 antibody to ENO1 used in the previous report (21) has not been seen in any published data, we suspect that 9G12 could be typing error for 9C12. The 9C12 antibody, originally developed from Redlitz's laboratory (8), recognizes both 54-kDa and 48-kDa proteins in U937 lysates and ENO1 purified from human brain, respectively, indicating that this antibody may recognize an unidentified  $\alpha$ -enolase-related molecule besides ENO1. Furthermore, its cross-reactivity to ENO2 is not clear because ENO1 epitope sequence of 9C12 antibody (42) shares 81.3% protein identity with human ENO2 protein. ENO2 ( $\gamma$ -enolase) not only abundantly exists in neural and bronchopulmonary neuroendocrine cells (43) but also expresses in normal nonneuron tissues (44), including bronchial and type II alveolar epithelial cells.

To further explore if existence of distinct amount of ENO2 in lung carcinoma might account for different outcomes between two laboratories, we adopted Western blotting analysis of two pairs of normal and tumor counterpart lysates for detection of ENO-1, ENO-2, and ENO-3 expressions. Antibody specificity to each isoform of enolases was shown in Fig. 2B. Using those antibodies, a preliminary study clearly showed one of the two tumor lysates displaying significant down-regulation of ENO2 compared with that in its normal counterpart (Supplementary Fig. S4). In contrast, ENO1 is still consistently up-regulated in both tumor parts. Therefore, isoform specificity of antibody used for detection of ENO1 becomes a key issue to correctly interpret resultant clinical outcomes of cancer patients.

Changes in energy metabolism are fundamental properties of cancer cells by which the cells survive in a state of hypoxic stress followed by promotion of angiogenesis and enhancement of local invasion or distal metastasis. In this study, up-regulation of glycolytic enzymes, such as ENO1, in NSCLC patients is highly associated with poorer clinical prognoses for tumor recurrence and survival. Coincidentally, surface ENO1 also detected in the effusion tumor cells may implicate its potential role in cell invasion. Using a low-invasive cell line and its invasive counterpart, we preliminarily support this implication.<sup>15</sup> Conclusively, we developed an antibody and showed its specificity exclusive to ENO1 in this study. Using this antibody, we show that increasing expression of ENO1 is a prevailing phenomenon in patients with NSCLC and its expression status is tightly correlated with disease recurrence and survival. Hence, ENO1 can be a good prognostic indicator to monitor disease progression for NSCLC patients, but also, its surface-localized antigen may serve as a target of immunotherapy for cancers in future.

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