WWOX Expression in Different Histologic Types and Subtypes of Non – Small Cell Lung Cancer

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Abstract

Purpose: Non – small cell lung cancer (NSCLC) has heterogeneous histopathologic classification and clinical behavior and very low survival rate. *WWOX* (WW domain-containing oxidoreductase) is a tumor suppressor gene, and its expression is altered in several cancers. The purpose of this study is to better define the role of WWOX in NSCLC tumorigenesis and progression by determining its pathogenetic and prognostic significance.

Experimental Design: WWOX protein expression was evaluated by immunohistochemistry in 170 patients with NSCLC (101 squamous cell carcinomas, 66 adenocarcinomas, 3 large cell carcinomas) and was correlated with histopathologic (histotype, subtype, grade, tumor-node-metastasis, stage, index of cell proliferation Ki67/MIB1) and clinical (age, gender, local recurrences, distant metastases, overall survival, and disease-free survival) characteristics.

Results: WWOX expression was absent/reduced in 84.9% of NSCLCs, whereas it was normal in 80.5% of adjacent normal lung tissues. WWOX expression was strongly associated with tumor histology ($P = 1.1 \times 10^{-5}$) and histologic grade ($P = 0.0081$): the percentage of cases with absent/strongly reduced WWOX expression was higher in squamous cell carcinomas and in poorly differentiated tumors. Regarding adenocarcinoma, bronchioloalveolar pattern showed normal WWOX expression in 62.5% of the cases, whereas in solid and acinar patterns, a prevalence of cases with absent/very low WWOX expression was observed (79.2% and 50%, respectively). Finally, weak WWOX staining intensity was related to the high index of cell proliferation ($P = 0.0012$).

Conclusions: Our results suggest that the loss of WWOX expression plays different roles in tumorigenesis of distinct histotypes and subtypes of NSCLC and is related to high aggressiveness (G_3; high proliferating activity) of tumors.

Lung cancer is the most common cancer and the biggest killer among malignancies in both men and women throughout the world today, representing 12.6% of new cancers and causing 17.8% of cancer death (1). Lung cancer is peculiar for its significant heterogeneity in terms of both histopathologic classification and clinical behavior. As far as lung cancer histopathology is concerned, in addition to the general classification of non – small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), several histologic types and subtypes of NSCLC have been described. NSCLC, which accounts for 80% of lung cancers, includes three major histotypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. On its own, adenocarcinoma, which has surpassed squamous cell carcinoma as the most common histologic type of NSCLC (1), may consist of individual or mixed distinct histologic patterns/subtypes, such as acinar, papillary, bronchioloalveolar, solid, clear cell, mucinous, and signet ring. Adenocarcinomas with bronchioloalveolar features, which can be divided into mucinous (type I), nonmucinous (type II), and mixed subtypes, constitute a distinct clinical, radiological, and pathologic entity among adenocarcinomas and have a peculiar epidemiology because they affect a higher percentage of women and nonsmokers (2, 3). The histologic heterogeneity of lung cancer likely reflects differences in cell derivation, genetic alterations, and pathogenetic pathways associated with tumor progression and may be responsible for fundamental discrepancies in tumor biology determining poor outcomes of lung cancer. In fact, in contrast to other

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common tumors, such as breast, prostate, and colorectal carcinoma, in which survival rates have dramatically improved because of innovations in early detection and in surgical and medical treatments during the past three decades, the clinical behavior of NSCLC remains bad; even patients with early-stage disease develop local recurrences and distant metastases or eventually die in about 30% of the cases within 5 years after complete surgical resection, which is currently the only potentially curative treatment for NSCLC (1). Moreover, the best predictor of survival for patients with lung cancer is still the tumor stage according to the tumor-node-metastasis classification (4). For these reasons, NSCLC represents a major focus for molecular classification, and many investigators are trying to find specific "profiles" or "gene expression signatures" of different histotypes and subtypes to add further prognostic information, select high-risk patients for aggressive adjuvant treatments, and set new "tailored" anticancer therapies.

WWOX (WW domain-containing oxidoreductase) is a recently cloned tumor suppressor gene (5) and is altered at the genomic and expression level in several types of cancer, including breast (6 – 8), ovarian (9), prostate (10), hepatocellular (11), pancreatic (12), esophageal (13), and gastric (14) carcinoma. The *WWOX* gene spans the second most active common fragile site in the human genome (FRA16D) at chromosome region 16q23.2 (5, 15, 16). Over the last few years, it has become clear that genes at common fragile sites are frequently inactivated early in the neoplastic process and are particularly susceptible to damage on exposure to environmental carcinogens, which are etiologic factors in lung cancer (17). Given these premises, in 2003, Yendamuri et al. analyzed 27 paired normal and tumor (10 adenocarcinomas, 11 squamous cell carcinomas, 4 poorly differentiated adenocarcinomas, and 2 tumors with other histologies) lung tissues and 8 lung cancer cell lines for *WWOX* alterations by reverse transcription-PCR, loss of heterozigosity, and mutation analysis (18). Transcripts missing *WWOX* exons were described in 7 out of 27 (25.9%) primary tumors and in 5 out of 8 (62.5%) cell lines; *WWOX* allele loss occurred in 36.4% (4 out of 11) of squamous cell carcinomas and in 30.0% (3 out of 10) adenocarcinomas (18). Recently, Iliopoulos et al. observed that *WWOX* altered expression in lung, breast, and bladder cancers is due not only to genomic alterations, such as loss of heterozygosity and homozygous deletions, but also to epigenetic modifications, such as promoter hypermethylation. *WWOX* promoter hypermethylation was observed in 62.5% of squamous cell lung carcinomas and seems to show differential patterns in neoplastic versus adjacent non-neoplastic tissues (19).

Others and we have shown that WWOX behaves as a tumor suppressor. It is a recent evidence that the restoration of WWOX expression, through recombinant adenovirus infection or through drug-inducible system, in lung cancer cells lacking the expression of endogenous WWOX, induces apoptosis *in vitro* and dramatically suppresses tumorigenicity in athymic nude mice (20).

Because no systematic studies correlating WWOX protein expression to tumor and patients characteristics in NSCLC have yet been reported, we set to determine whether WWOX might have a pathogenetic and prognostic role in NSCLC. We evaluated WWOX protein expression, as assessed by immunohistochemistry, in a large series (170 cases) of stages I to IIIA NSCLC and adjacent normal lung tissue, when present, and correlated it with histologic and clinicopathologic characteristics. We found that WWOX expression is deleted or reduced in the vast majority of NSCLCs, and that loss of WWOX expression is strongly related to high aggressiveness (high histologic grade, G_3; high index of cell proliferation) of tumors. In addition, we found that WWOX expression is strongly correlated with tumor histology, thus suggesting that the loss of WWOX expression plays different roles in tumorigenesis of distinct histotypes and subtypes of NSCLC.

Materials and Methods

Patients and clinical data. A total of 170 patients with NSCLC, who consecutively underwent radical surgical resection at the Department of Cardio-Thoracic Surgery of the University of Pisa from December 1991 to December 1994, were studied. Participation in the study required informed consent. No detectable metastases in distal organs were present at the time of surgery. No patient had received chemotherapy nor radiotherapy before surgery. Follow-up lasted through June 30, 2003, with a median follow-up period of 49.5 months for living patients (range, 2-137 months). Clinical parameters, such as patient gender, age, local recurrences, distant metastases, and disease-free and overall survival, were reviewed for each patient. Disease-free survival and overall survival rates were calculated as the period from surgery until the date of disease relapse and of death, respectively.

Specimens. At least four samples of neoplastic tissue from each tumor were removed, depending on the tumor size and the presence of regressive alterations. Neoplastic specimens were always removed from the periphery of the tumor masses because the central region of a cancer is more often subject to regressive alterations, such as necrosis and hemorrhage. The tumor samples were formalin fixed and paraffin embedded for histologic and immunohistochemical analysis. The most representative paraffin block of tumor, including adjacent normal tissue when possible, was selected for each case. The pathologic features (histologic type and histologic grade) of each tumor, classified at the time of surgery, were reevaluated by two pathologists (V. Donati and G. Fontanini) according to the WHO 2004 histologic criteria (1). Particular attention was paid to recognize different histologic subtypes/patterns (acinar, papillary, bronchioloalveolar mucinous/nonmucinous/mixed, solid, mixed, clear cell, signet ring, mucinous, or "colloid") of adenocarcinoma. Tumor staging was done according to the International Union Against Cancer tumor-node-metastasis classification (4).

Immunohistochemistry. WWOX expression was detected by immunohistochemistry using a polyclonal rabbit anti – glutathione-*S*-transferase (anti-GST)-WWOX antibody (diluted at 1:4,000; ref. 6). The antibody was applied to sections from the most representative formalin-fixed, paraffin-embedded tumor blocks obtained from each of the 170 patients with NSCLC, using the avidin-biotin-peroxidase complex method (Vectastatin Elite ABC Kit Rabbit IgG; Vector Laboratories, Inc.; Burlingame, CA), and following the manufacturer's instructions. The immunostaining was done manually at room temperature. Sections of 5 μm, mounted on glass slides, were deparaffinized through serial baths in xylene and rehydrated in a graded series of alcohol and water. Antigen unmasking was done by heating sections at boiling temperature in preheated 1× Target Retrieval Solution (DakoCytomation, Glostrup, Denmark) for 30 min, using a steamer. After cooling sections in solution at room temperature for 20 min and washing them in TBS thrice, sections were soaked in absolute methanol containing 0.3% hydrogen peroxide for 30 min at room temperature to remove any endogenous peroxidase activity and nonspecific background staining. After being washed with TBS for 5 min, slides were blocked with nonimmune goat serum for 30 min to inhibit nonspecific binding. This step was followed by incubation with the anti-WWOX primary antibody for 60 min at room temperature.

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After rinsing with TBS for 5 min, sections were subsequently incubated with biotinylated goat anti-rabbit secondary antibody for 30 min. Then, after being washed again with TBS for 5 min, slides were incubated with avidin-biotin-peroxidase complex for 30 min and washed again with TBS. Finally, the sections were incubated with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Vectastain; Vector Laboratories) and then rinsed in distilled water. All slides were lightly counterstained with Mayer's hematoxylin for 30 s, washed in running water, dehydrated, and mounted with Canadian balsam. A section of normal human breast, previously proven to be WWOX positive, was used as positive control, whereas an invasive ductal carcinoma case that was negative for WWOX protein expression was included as negative control.

Two pathologists (V. Donati and G. Fontanini), who were blinded to clinical information, evaluated and scored immunohistochemical stains for WWOX protein in both tumor tissue and adjacent normal tissue, when present. Immunohistochemical staining was scored taking into account both staining intensity and staining extent. Intensity of staining was graded as follows: lost (score 0); weak ("+"; score 1); moderate ("++"; score 2); and strong ("+++"; score 3). Extent of staining was evaluated as the percentage of cells with cytoplasmic immunoreactivity counting at least 1,000 cells (100 cells in 10 high-power fields) in each evaluated compartment (tumor tissue and normal tissue) and was graded in six classes as: negative (score 0); ≤10% (score 1); 11% to 25% (score 2); 26% to 50% (score 3); 51% to 75% (score 4); >75% (score 5). A final staining score was calculated by multiplying intensity and extent scores for every compartment, and we distinguished three classes: one with normal expression of WWOX (final score "A": 10-15); one with reduced expression of WWOX (final score "B": 5-9); one with absent or very low expression of WWOX (final score "C": 0-4; Figs. 1 and 2). For adenocarcinomas with more than one pattern (mixed subtype), the staining score of each pattern was determined and analyzed separately.

In a selected subgroup of tumors (33 squamous cell carcinomas; 23 adenocarcinomas, 8 of which with mixed subtype; 2 large cell carcinomas), we assessed the index of cell proliferation by immunohistochemistry using an anti-Ki-67 monoclonal antibody (clone MIB1; DBA, Milan, Italy) at a dilution of 1:100. The immunohistochemical staining for Ki-67 was evaluated as the percentage of cancer cells with nuclear immunoreactivity counting at least 1,000 tumor cells per slide (magnification, ×40). The median value of this series (34.85% of positive cells) was used as the cutoff value to distinguish tumors with low (<34.85%) from tumors with high (≥34.85%) index of cell proliferation.

Statistical analysis. Statistical analysis was carried out using R 2.2.0 (21). Univariate analysis was done by modelling Kaplan-Meier survival curves. Log-rank test was used to evaluate the statistical significance of differences in survival distributions. Quantitative variables were categorized with respect to their median. χ^2 test was used to evaluate associations between score or staining of WWOX expression and clinicopathologic characteristics. All tests used are described in Armitage et al. (22). Results were considered statistically significant if $P < 0.05$.

Results

Clinicopathologic characteristics. Tumors were reevaluated and reclassified according to the WHO classification of tumours 2004 criteria (1): the most common histologic type was squamous cell carcinoma (59.4%; 101 cases), followed by adenocarcinoma (38.8%; 66 cases), and large-cell carcinoma (1.8%; 3 cases). Other histopathologic and clinical characteristics of the series of patients we studied are summarized in Table 1. Adenocarcinomas of the lung in our study group consisted purely of a single histologic pattern ("adenocarcinomas with single pattern") in 59.1% (39 out of 66) of the cases, whereas they were "mixed subtypes" in the remaining 40.9% (Table 2).

WWOX ***expression in NSCLC and adjacent normal tissue.*** WWOX expression was assessed in each tumor and, when possible (72 cases), in the adjacent normal lung tissue,

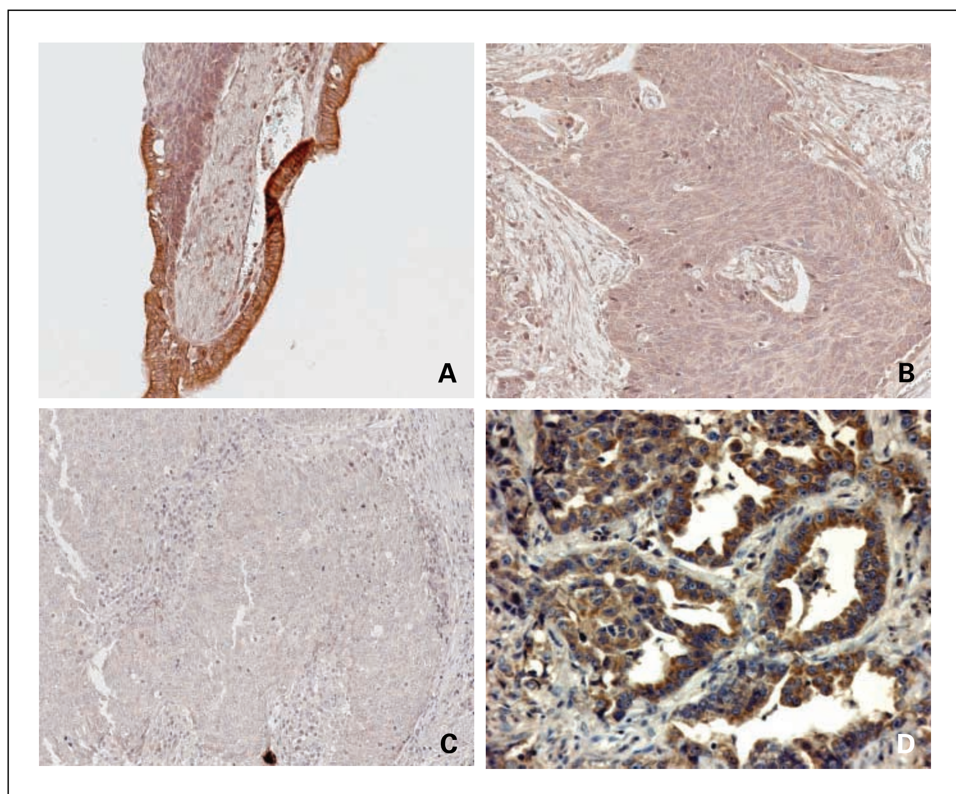

Fig. 1. Differences in WWOX expression between non – small cell lung cancer tissue (*B*) and adjacent normal lung tissue (*A*) from the same patient, and between different histologic types of non – small cell lung cancer (*C* and *D*). *A* and *B,* a case with normal expression of WWOX in normal lung tissue (bronchial epithelial cells; *A*) and very low expression of WWOX in lung tumor (squamous cell carcinoma; *B*). *A,* normal lung tissue: strong intensity (+++, score 3) × extent >75% (>75%, score 5) = normal WWOX expression (total staining score A, 10-15). *B,* squamous cell carcinoma: weak intensity (+, score 1) × extent 51% to 75% (51-75%, score 4) = very low expression of WWOX (total staining score C, 0-4). *C,* a WWOX-negative squamous cell carcinoma of the lung. *D,* normal expression of WWOX in an adenocarcinoma of the lung with acinar pattern: moderate staining intensity of WWOX (++, score 2) × extent >75% (>75%, score 5) = total staining score A (10 – 15). *A-D,* original magnification, ×100.

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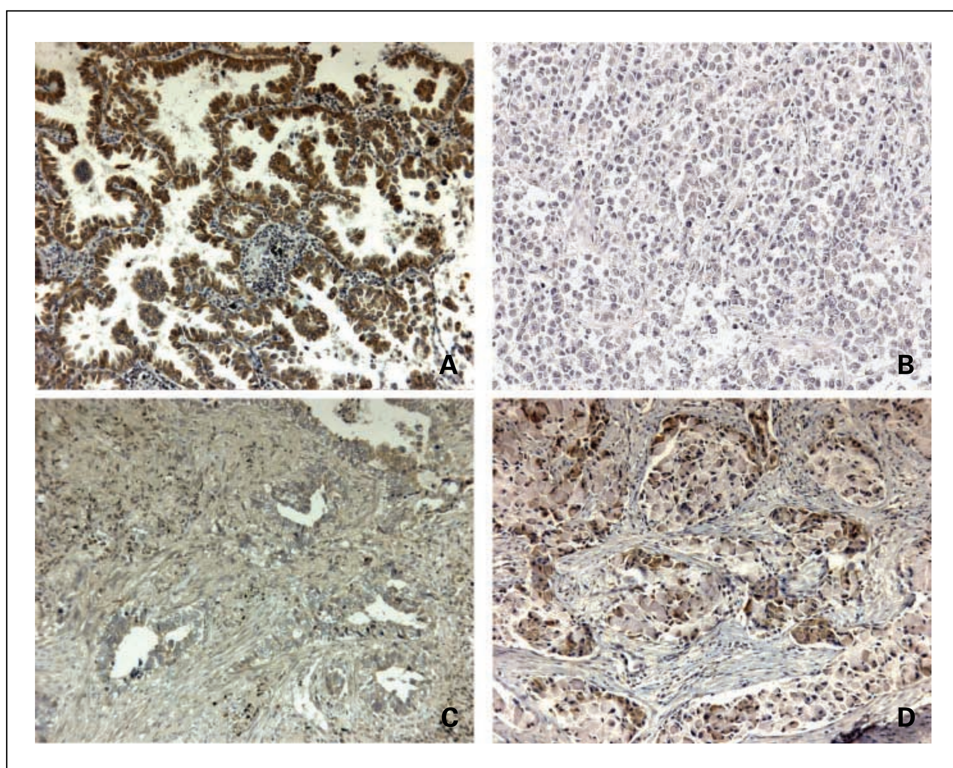

Fig. 2. Differences in WWOX expression between distinct histologic subtypes of adenocarcinoma of the lung (*A-D*). *A,* an example of adenocarcinoma of the lung with nonmucinous bronchioloalveolar pattern with intense positive staining of WWOX (+++, score 3) in >75% (>75%, score 5) of tumor cells. *B,* a WWOX-negative adenocarcinoma of the lung with solid pattern. *C,* loss of WWOX expression in an adenocarcinoma of the lung with acinar pattern: weak staining (+) in ≤10% of tumor cells. *D,* adenocarcinoma of the lung with signet ring subtype with moderate cytoplasmic staining of WWOX (++) in 11% to 25% (11-25%) of tumor cells. Original magnification: *B-D,* ×100; *A,* ×200.

bronchial epithelial cells and alveolar macrophages. According to the total staining scores, WWOX expression was lost or reduced in 84.9% of tumors (score C: 67.3%; score B: 17.6%), whereas it was normal in 80.5% (58 out of 72 cases) of adjacent normal tissues. About 15.6% of the tumors were WWOX negative, and 56.3% of them had a weak staining intensity of WWOX, whereas in only 12.5% (9 out of 72) of normal tissues, the staining intensity of WWOX was low. Focusing on adjacent normal lung tissues, WWOX protein was expressed in the cytoplasm of both normal bronchial epithelial cells and alveolar type II cells, with no significant differences in WWOX expression among these two distinct normal cell types. None of the examined normal lung tissues was WWOX negative. WWOX staining intensity was "strong" (+++) in 43.1% (31 out of 72) of normal lung cells and "moderate" (++) in 44.4% (32 out of 72) of them. Focusing on paired tumor and normal tissues, in most cases (80.5%), the staining scores for tumors were less (Fig. 1B) than the scores in normal tissues (Fig. 1A); in 18.1% of patients, normal tissue and lung cancer had the same staining scores; and in only 1 case (1.4%), the staining score for cancer was higher than the score in normal tissue (cancer with score A versus normal with score C). Among the 13 patients with the same staining scores for normal tissue and lung cancer, 8 had normal expression of WWOX (score A), whereas 5 showed lost or very low WWOX expression (score C). Interestingly, in seven out of the eight patients with normal expression of WWOX both in tumor and in normal tissue, and in the only patient with WWOX expression higher in tumor than in normal lung, the histologic type of the tumors was adenocarcinoma.

WWOX ***expression and histologic types of NSCLC.*** In our series of NSCLC, WWOX expression was strongly related to the histologic type (Fig. 1C and D; total staining score, $P = 1.1 \times 10^{-5}$, Table 3A; intensity of staining, $P = 0.00066$, Table 3B; extent of staining, $P = 0.00130$). In fact, according to the total staining scores, the percentage of tumors with loss or strong reduction (score C) of WWOX expression was higher in squamous cell carcinomas (80.2%) than in adenocarcinomas (52.6%), and all large cell carcinomas (three cases) were scored as C (Fig. 3A). Focusing on WWOX staining intensity and histotypes, in only 15.8% of squamous cell carcinomas, WWOX staining intensity was strong (2 cases out of 101) or moderate (14 cases out of 101), compared with 42.1% of adenocarcinomas (data not shown).

WWOX ***expression and histologic subtypes of adenocarcinoma of the lung.*** Focusing on adenocarcinoma, a statistically significant association between its different histologic subtypes/patterns and WWOX expression (Fig. 2A-D), evaluated both as total staining score and as intensity of staining, was observed (total staining score, $P = 4.5 \times 10^{-5}$, Table 3A; intensity of staining, $P = 6.8 \times 10^{-5}$, Table 3B). According to the total staining scores, adenocarcinomas with bronchioloalveolar pattern showed normal WWOX expression (score A; Fig. 2A) in 62.5% of the cases, whereas in adenocarcinomas with solid (Fig. 2B) and acinar (Fig. 2C) patterns, a prevalence of cases with absence or strong reduction in WWOX expression (score C) was observed (79.2% and 50%, respectively; Fig. 3B). Almost all adenocarcinomas with solid subtype (23 out of 24) showed loss (0) or weak (+) intensity of WWOX staining.

WWOX ***expression and histologic grade of NSCLC.*** A strongly significant correlation between poorly differentiated (G_3) tumors and absent or highly reduced WWOX expression was observed (total staining score, $P = 0.0081$, Table 3A; extent of staining, $P = 0.014$). In fact, according to the total staining score, the percentage of tumors with loss or high reduction (score C) of WWOX expression was higher in G_3 (76.6%) than in well-differentiated (60.0%) tumors (Fig. 3C).

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Table 1. Clinicopathologic characteristics of 170 patients with non–small cell lung cancers (study group)

Clinicopathologic characteristics	Proportion
Gender	
Male	90%
Female	10%
Age	
Median age (y)	64.5
Range (y)	41-88
Histology	
Squamous cell carcinoma	59.4%
Adenocarcinoma	38.8%
Large cell carcinoma	1.8%
Grade (G)	
Well differentiated, G_1	23.5%
Moderately differentiated, G_2	39.8%
Poorly differentiated, G_3	36.7%
Tumor size (T)	
T1	28.8%
T2	61.8%
T3	9.4%
Lymph node metastases (N)	
Absent, N0	67.1%
Hilar, N1	18.2%
Mediastinal, N2	14.7%
Stage (S)	
S I	62.9%
S II	14.7%
S IIIA	22.4%
Relapses	48.8%
Distant metastases	77.1%
Local recurrences	22.9%
Status	
Alive	52.9%
Dead	47.1%

WWOX ***expression and other clinicopathologic characteristics.*** Associations between WWOX expression and other clinicopathologic parameters were analyzed on the whole cohort of patients, on patients with early stage (I) versus patients with advanced stage (II and IIIA) of disease, on patients with squamous cell carcinoma versus patients with non-squamous cell carcinoma, and on patients with different histologic patterns of adenocarcinoma. WWOX expression, evaluated as total staining score (Table 3A), staining intensity (Table 3B), and staining extent (data not shown), did not show any statistically significant correlation with gender, age, tumor size (T), lymph node metastases (N), and stage (S). In the survival analyses, WWOX expression turned out not to be related to disease-free survival nor overall survival.

WWOX ***expression and index of cell proliferation (Ki-67).*** In our study group, a strong correlation between loss of WWOX expression and high index of cell proliferation was described (P = 0.0012, Table 3B). In fact, WWOX expression was completely lost or very weak in 93.9% of tumors with high expression of MIB1-Ki-67 proliferating-cell antigen (Fig. 3D).

Discussion

Lung cancers result from complex genetic and epigenetic changes characterized by stepwise malignant progression of cancer cells in association with accumulation of genetic alterations (1). Evidence has suggested that, although some genetic changes occur independently of the histologic type, their frequency and timing of occurrence with respect to cancer progression is different in distinct histotypes. For example, although the LOH on chromosome 3p is a genetic change common in different histologic types of NSCLC, differences in the loss of heterozygosity on 3p between squamous cell carcinoma and adenocarcinoma have been described: squamous cell carcinomas show larger 3p segments of allelic loss than adenocarcinomas (23). Chromosome 3p encompasses several potential tumor suppressor genes, including the *FHIT* (fragile histidine triad) gene, which is a well-studied gene in lung cancer. *FHIT* is located in a highly fragile chromosomal site (FRA3B), which has been found to be particularly prone to direct DNA damages by carcinogens present in tobacco smoke (24, 25). If tumor suppressor genes encompass fragile sites, damages by carcinogens in these sites may provide an etiologic mechanism for carcinogenesis. This evidence could explain the different incidence of squamous cell carcinomas and adenocarcinomas related to tobacco smoke (tobacco smoking increases the risk of all major histologic types of lung cancer, but seems to be strongest for squamous cell carcinoma).

WWOX, like *FHIT*, is located at a common fragile region (FRA16D) and behaves as a tumor suppressor gene. Many evidences suggest that *FHIT* and *WWOX* are likely to be coordinately inactivated in cancer (6, 14). In lung cancer, as in breast and bladder carcinomas, *WWOX* and *FHIT* abnormalities are due not only to genomic alterations, such as loss of heterozygosity and homozygous deletions, but also to epigenetic modifications (19).

To our knowledge, in NSCLC, no extensive study correlating WWOX protein expression to histopathology (squamous cell carcinoma, adenocarcinoma, large cell carcinoma; different patterns of adenocarcinoma) and to tumor (histologic grade, tumor-node-metastasis, stage, index of cell proliferation) and patients (gender, age, local recurrences, distant metastases, disease-free survival, and overall survival) characteristics has

Table 2. Histologic subtypes of adenocarcinomas of the lung

Histology of adenocarcinomas	Patterns	Number of cases
Adenocarcinomas with single pattern		39
	Bronchioloalveolar	14
	Nonmucinous	9
	Mucinous	4
	Mixed	1
	Acinar	13
	Solid	9
	Mucinous	1
	Signet ring	1
	Clear cell	1
Adenocarcinomas mixed subtypes		27
	Solid and acinar	12
	Acinar and bronchioloalveolar	6
	Acinar and papillary	4
	Solid and bronchioloalveolar	2
	Acinar and mucinous	1
	Acinar and bronchioloalveolar and signet ring	1
	Solid and acinar and bronchioloalveolar	1

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Table 3. Associations between staining score and intensity of WWOX and clinicopathologic characteristics

(A) Associations between total staining score of WWOX expression and clinicopathologic characteristics

	P value
Gender	0.11
Histologic type	1.1×10^{-5} (Fig. 3A)
Adenocarcinoma subtypes	4.5×10^{-5} (Fig. 3B)
T	0.48
N	0.19
Stage	0.062
G	0.0081 (Fig. 3C)
MIB1	0.059

(B) Associations between WWOX staining intensity and clinicopathologic parameters

	P value
Gender	0.34
Histology	0.00066
Pattern	6.8×10^{-5}
T	0.68
N	0.82
Stage	0.19
G	0.12
MIB1	0.0012 (Fig. 3D)

NOTE: *P* values were obtained by performing the Fisher's exact test.

been conducted. We evaluated by immunohistochemistry WWOX protein expression in 170 cases of NSCLC, and in 72 out of them, it has been possible to determine WWOX protein expression also in the adjacent normal lung tissue. In accordance with previous reports (6, 19), we used three different parameters to estimate WWOX immunoreactivity. We examined correlations with the extent of staining and the intensity of the staining, but because in a high percentage of tumors the staining pattern was heterogeneous, we observed that a total staining score, which was the result of multiplication of extent and intensity, was the best method to estimate WWOX immunoreactivity.

In 84.9% of our series of NSCLC, WWOX expression was absent or reduced, whereas it was normal in 80.5% of adjacent normal lung tissues. Interestingly, WWOX expression was strongly related to the histologic type (total staining score, $P = 1.1 \times 10^{-5}$; intensity of staining, $P = 0.00066$; extent of staining, $P = 0.00130$). Squamous cell carcinomas showed absence or down-regulation of WWOX expression in a proportion of tumors (80.2%) that was higher than that of adenocarcinomas (52.6%). This evidence could be related to DNA abnormalities at fragile sites induced by tobacco smoke, and consequently, to the higher risk of developing squamous cell carcinoma rather than adenocarcinomas in smokers. Interestingly, we noticed that the percentage of tumors with negative or weak staining intensity was much higher in squamous cell carcinomas (84.2%) than in adenocarcinomas (57.9%). This evidence suggests that WWOX inactivation occurs at different times with respect to cancer progression in these two histotypes. Because many squamous cell carcinomas show a relatively high extent of staining, but a very low intensity of staining, it is likely that WWOX inactivation is an early event in squamous cell carcinomas; on the other hand, given the fact that a fraction of adenocarcinomas exhibits WWOX staining patterns characterized by moderate-strong staining intensity, but not so high staining extent, we speculate that in these tumors, selective tumor clones lose WWOX expression in later stages. Another interesting observation supporting the different role of WWOX in squamous cell carcinomas and adenocarcinomas pathogenesis is that in seven out of the eight patients with normal expression of WWOX both in tumor and in normal tissue, and in the only patient with WWOX expression higher in tumor than in normal lung, the histologic type of the tumors was adenocarcinoma. Our results thus indicate that WWOX may play distinct roles in different histotypes of NSCLC.

Adenocarcinoma is at present the histologic type of non–small cell lung carcinoma where the work of gene expression profiling as a powerful tool for molecular classification is most advanced. Several subtypes of adenocarcinoma with different behavior and prognosis have been described. The major

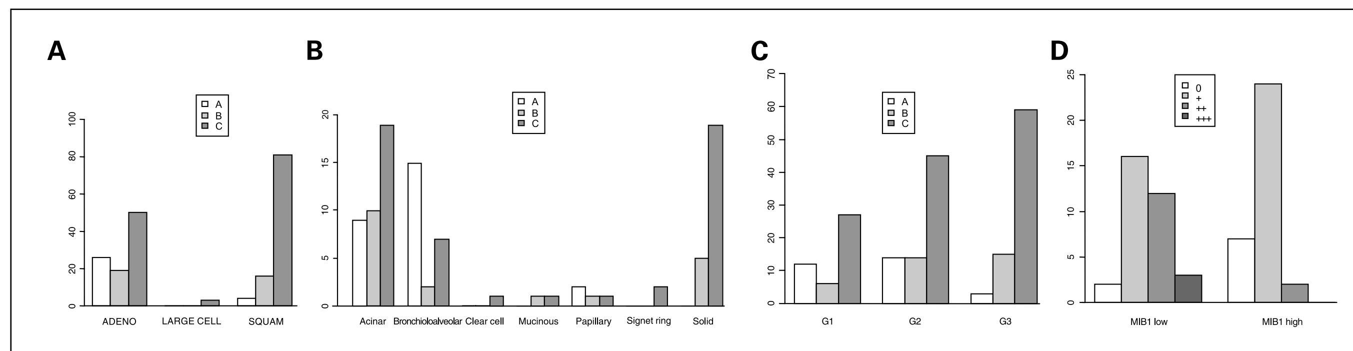

Fig. 3. Classification of our study group of NSCLC based on WWOX total staining scores and (*A*) histologic types, (*B*) histologic subtypes of adenocarcinoma of the lung, (*C*) histologic grade, and (*D*) on WWOX staining intensity scores and index of cell proliferation (Ki-67/MIB1). *A, X-axis,* the three major histologic types of NSCLC (*ADENO,* adenocarcinomas; *LARGE CELL,* large cell carcinomas; *SQUAM,* squamous cell carcinomas); *Y-axis,* the number of cases relative to the total staining scores (A, B, or C). *B, X-axis,* different histologic subtypes of adenocarcinoma of the lung (acinar, bronchioloalveolar, clear cell, mucinous, papillary, signet ring, and solid); *Y-axis,* the number of cases relative to the total staining scores (A, B, or C). *C, X-axis,* different tumor histologic grades (G_1, well differentiated; G_2, moderately differentiated; G_3, poorly differentiated); *Y-axis,* the number of cases relative to the total staining scores (A, B, or C). *D,* the immunohistochemical staining for Ki-67 was evaluated as the percentage of cancer cells with nuclear immunoreactivity counting at least 1,000 tumor cells per slides (×40 magnification). *X-axis,* the median value of this series (34.85% of positive cells) was used as cutoff value to distinguish tumors with low (<34.85%) from tumors with high (≥34.85%) index of cell proliferation (*MIB1 low* versus *MIB1 high*); *Y-axis,* the number of cases relative to the scores of WWOX staining intensity (0, +, ++, or +++).

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individual histologic patterns are acinar, papillary, solid, and bronchioloalveolar. The papillary pattern seems to represent an unfavorable prognostic finding (26–28), whereas adenocarcinomas with a predominant bronchioloalveolar pattern and central scarring <0.5 cm in tumors of 3 cm or less or pT1 tumors have a very favorable prognosis (29–31). In the last few years, the definition of bronchioloalveolar carcinoma has been subject to important changes. The WHO has redefined bronchioloalveolar carcinoma as an adenocarcinoma variant that grows along preexisting alveolar structures without evidence of stromal, vascular, or pleural invasion (32, 33); in case of invasive component, the tumor must be called "adenocarcinoma with bronchioloalveolar pattern." As a consequence of this change of definition, we reclassified many cases previously defined bronchioloalveolar carcinomas as adenocarcinomas with bronchioloalveolar pattern. In our study, according to the total staining scores, adenocarcinomas with bronchioloalveolar pattern showed normal WWOX expression in 62.5% of the cases, whereas in adenocarcinomas with solid and acinar patterns, a prevalence of cases with the absence or strong reduction in WWOX expression was observed (79.2% and 50%, respectively). Almost all adenocarcinomas with solid subtype showed loss or weak intensity of WWOX staining. Recently, Nunez et al. observed that WWOX protein expression varies among ovarian carcinoma histotypes. Interestingly, they described a significant loss of WWOX expression in mucinous (70%) and clear cell (42%) ovarian carcinomas, which are two rare but well-described ovarian carcinoma histotypes with distinct molecular profiles: mucinous ovarian carcinomas are characterized by activating K-ras mutations, whereas clear cell carcinoma show a lack of p53 mutations (34). Among adenocarcinomas of the lung, K-ras oncogene activation by point mutations (mostly in codon 12) is present in a significant percentage (30-40%) of cases and correlates with poor survival (35–37). Ras-dependent pathway can be initiated by the activation of two important tyrosine kinase receptors belonging to the same family, the epidermal growth factor receptor (EGFR or ErbB-1) and HER-2/neu (ErbB-2). EGFR and, to a less extent, HER-2/neu are overexpressed in NSCLCs (38). EGFR is overexpressed in a high proportion (80%) of squamous cell carcinoma (39), and somatic heterozygous missense mutations in its exons 18 to 21, which encode for the tyrosine kinase domain, are significantly more frequent in adenocarcinomas, in never-smokers, and in female gender (40, 41). Very interestingly, the presence of somatic presumably activating mutations in the tyrosine kinase domain of the EGFR gene in NSCLC predicts for response to EGFR tyrosine kinase inhibitors (40, 41), which are selective reversible inhibitors that compete with ATP for binding to the tyrosine kinase domain and abrogate the receptor's catalytic activity. HER-2/neu is an important target in breast cancer therapy and, although its overexpression in lung cancer is not so frequent, could be a potential adjunctive target in the therapy of adenocarcinoma of the lung, where it is occasionally overexpressed. Moreover, ErbB-2 is the preferential heterodimerization partner for EGFR. EGFR and HER-2/neu are members of the ErbB family of tyrosine kinase receptor proteins, which also includes ErbB-3 (HER-3) and ErbB-4 (HER-4; ref. 42). We have recently reported that WWOX competes with YAP (Yes-associated protein) for binding to the intracellular domain of ErbB-4 and, by sequestering the latter in the cytoplasm, suppresses its transcriptional ability (43). A recent study has reported that ectopic expression of ErbB-4 in a human NSCLC cell line, which did not express the ErbB-4 protein, resulted in an increased cell proliferation *in vitro* and *in vivo*, and that a monoclonal antibody to ErbB-4 showed both an inhibitory effect on growth rate and an increasing apoptotic rate in the cells expressing ErbB-4 (44). Interestingly, Tal-Or et al. recently observed that Ras induces ErbB-4 receptor phosphorylation in a ligand-independent manner, and that this activation depends on multiple Ras effector pathways and on ErbB-4 kinase activity (45). Given these premises, an interesting future prospective could be the study of EGFR, HER-2/neu, HER-4, and Ras expression in those cases with loss of WWOX expression to define WWOX pathway.

Finally, we described a very strong correlation between loss or strong reduction of WWOX expression and poor histologic differentiation (G_3) of tumors, as well as between the lack of WWOX and high index of cell proliferation. Our results suggest that WWOX absence or down-regulation correlates with an aggressive biological behavior of tumors. Moreover, because recent evidences have proved that WWOX suppresses tumor growth *in vitro* or *in vivo*, as well as transcriptional activity of putative proto-oncogenes such as ErbB4, the observation that tumors with absent or very weak WWOX expression had high index of cell proliferation represents a further evidence that WWOX expression is related to tumor proliferation, and in particular, that tumors are highly proliferating when WWOX is lost or down-regulated. Indeed, WWOX overexpression induces caspase-mediated apoptosis in cancer cells lacking expression of endogenous WWOX (20). In addition, the fact that WWOX associates with transcription factors involved in cell cycle regulation, such as p73, p53, and AP2γ (46–48), suggests that loss of WWOX may cause cell cycle deregulation, a hallmark of cancer phenotype.

In conclusion, WWOX expression differs among individual histologic types of NSCLC and among distinct patterns of adenocarcinoma of the lung, suggesting that WWOX plays a different role in these cancers pathogenesis, and that it could be used as a marker useful to a more accurate subclassification of NSCLC. Moreover, because loss or high reduction of WWOX expression is mainly observed in poorly differentiated (G_3) and high proliferating NSCLCs, and given the fact that WWOX functions as a tumor suppressor in preclinical lung cancer models, our results confirm that WWOX could be an important target for tailored therapies.

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