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Clinical Cancer Research

Imaging, Diagnosis, Prognosis

EZH2 Protein Expression Associates with the Early Pathogenesis, Tumor Progression, and Prognosis of Non–Small Cell Lung Carcinoma

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Abstract

Purpose: Enhancer of zeste homolog 2 (EZH2) promotes carcinogenesis by epigenetically silencing tumor suppressor genes. We studied EZH2 expression by immunohistochemistry in a large series of non–small cell lung carcinomas (NSCLC) in association with tumor characteristics and patient outcomes.

Experimental Design: EZH2 immunohistochemistry expression was analyzed in 265 normal and premalignant bronchial epithelia, 541 primary NSCLCs [221 squamous cell carcinomas (SCC) and 320 adenocarcinomas] and 36 NSCLCs with paired brain metastases. An independent set of 91 adenocarcinomas was also examined. EZH2 expression was statistically correlated with clinico-pathological information, and *EGFR*/*KRAS* mutation status.

Results: EZH2 expression was significantly ($P < 0.0001$) higher in SCCs compared with adenocarcinomas and in brain metastasis relative to matched primary tumors ($P = 0.0013$). EZH2 expression was significantly ($P < 0.0001$) elevated in bronchial preneoplastic lesions with increasing severity. In adenocarcinomas, higher EZH2 expression significantly correlated with younger age, cigarette smoking, and higher TNM stage ($P = 0.02$ to $P < 0.0001$). Higher EZH2 expression in adenocarcinoma was associated with worse recurrence-free survival (RFS; $P = 0.025$; HR = 1.54) and overall survival (OS; $P = 0.0002$; HR = 1.96). Furthermore, lung adenocarcinomas with low EZH2 levels and high expression of the lineage-specific transcription factor, TTF-1, exhibited significantly improved RFS ($P = 0.009$; HR = 0.51) and OS ($P = 0.0011$; HR = 0.45), which was confirmed in the independent set of 91 adenocarcinomas.

Conclusion: In lung, EZH2 expression is involved in early pathogenesis of SCC and correlates with a more aggressive tumor behavior of adenocarcinoma. When EZH2 and TTF-1 expressions are considered together, they serve as a prognostic marker in patients with surgically resected lung adenocarcinomas. *Clin Cancer Res; 19(23); 6556–65. ©2013 AACR.*

Introduction

Lung cancer is the most common cause of cancer-related death in the world (1). Non–small cell lung carcinoma (NSCLC) is the most common histological type of lung cancer with squamous cell carcinoma (SCC) and adenocarcinoma being the most prevalent subtypes (2). Despite intensive research, the prognosis of lung cancer patients remain poor, with an overall 5-year survival rate of 15% (1). For patients with early-stage disease, surgery is the mainstay of treatment (2). The development of clinically useful prognostic molecular markers is therefore crucial to identify subset of patients with a higher risk of recurrence and/or poor survival outcomes.

Enhancer of zeste homolog 2 (EZH2) is a key component of the polycomb repressive complex 2 (PCR2), which possesses histone methyltransferase activity and mediates gene silencing through posttranslational histone modifications (3). In addition, it also promotes cancer development and progression through chromatin modification by epigenetic activation of oncogenic signaling cascades and silencing of tumor suppressor genes, and has been implicated in cell proliferation, differentiation, invasion, and metastasis (3–5). Recently, it has been demonstrated in cells of castrate-resistant prostate cancer that EZH2 oncogenic function is independent of its role as a transcriptional

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Translational Relevance

Enhancer of zeste homolog 2, a potential molecular target of lung cancer, is frequently expressed in non–small cell lung carcinoma, and its expression associates with early pathogenesis and correlates with a more aggressive tumor behavior of lung adenocarcinomas.

repressor, and it would act as a coactivator for critical transcription factors (6). EZH2 is frequently overexpressed in a wide variety of human malignancies (7–12), including lung (13, 14), and it has been considered as a potential novel therapeutic target (5, 15, 16). In NSCLC, EZH2 protein overexpression has been associated with worse outcome in 2 relatively small series of patients with surgically resected tumors (13, 14, 17); however, the characterization of EZH2 expression in NSCLC by examining a large series of tumors with well-annotated clinical, pathological, and molecular information has not yet been reported.

In this study, we sought to determine the clinical relevance of EZH2 protein expression in a large ($N = 541$) series of surgically resected NSCLCs, including 221 SCCs and 320 adenocarcinomas. We studied the association of this protein with tumor histology and patients' clinico-pathologic features, including age, sex, stage, overall survival (OS), and recurrence-free survival (RFS) rates, and for adenocarcinomas with *EGFR* and *KRAS* mutation status of the tumors. To determine the expression of EZH2 in advanced metastatic NSCLC, we evaluated its expression in a series of 36 NSCLC lung primary and brain metastasis pairs. In addition, to investigate the relevance of EZH2 expression in the early pathogenesis of lung cancer, particularly SCCs, we examined its expression in a series of bronchial preneoplastic lesions.

Patients and Methods

Case selection

We collected formalin-fixed and paraffin-embedded (FFPE) tumor tissue from primary NSCLCs, including 221 SCCs and 320 adenocarcinomas from patients who had undergone surgical resection with curative intent between 1999 and 2005 at The University of Texas MD Anderson Cancer Center (Houston, Texas). This study was approved by the MD Anderson Cancer Center institutional review board. Patients' clinico-pathologic characteristics are shown in Table 1. None of these patients had received neoadjuvant therapy. Clinico-pathologic information was retrieved from the patients' electronic medical record and included age, sex, smoking status (current, former, or never), tumor size, tumor stage [according to the World Health Organization (18) and International Association for the Study of Lung Cancer (IASLC; ref. 19) classification systems], adjuvant treatment, and follow-up information (median 7.3 years for SCC and 6.7 years for adenocarcinoma) for OS and RFS rates. In addition, data on the histological patterns of adenocarcinoma were available. This has been previously described by Solis and colleagues (20). For the validation of the significant prognostic findings, we selected a smaller independent series of 91 patients with primary lung adenocarcinomas, surgically resected between 1996 and 2009 and with a median follow-up duration 4.8 years with similar clinical and pathological characteristics than first set of adenocarcinomas examined. To determine the expression of EZH2 in advanced metastatic NSCLC, we studied its expression in FFPE tissues from a series of 36 NSCLC (9 SCCs and 27 adenocarcinomas) lung primary tumors and their corresponding brain metastasis.

To investigate the relevance of EZH2 expression in the early pathogenesis of lung cancer, we examined its expression in bronchial epithelium FFPE tissues obtained from patients with surgically resected NSCLC, including normal epithelium ($N = 71$), hyperplasia ($N = 113$), squamous metaplasia ($N = 17$), low-grade dysplasia ($N = 17$), and high-grade dysplasia ($N = 113$).

Immunohistochemical analysis

To determine the immunohistochemical expression of EZH2 and TTF-1 in lung adenocarcinomas, we used FFPE tumors tissue placed in tissue microarray (TMA). The immunohistochemical analysis was performed using commercially available antibodies against EZH2 at 1:100 dilution (mouse monoclonal, NCL-L; Novocastra, Leica Niosystem), and TTF-1 at 1:200 dilution (mouse monoclonal, clone 8G7G3/1Dakocytomation). Immunohistochemical staining was performed using 5-μmol/L-thick sections from TMAs. Tissue sections were deparaffinized and hydrated, and antigen retrieval was performed in pH 6.0 citrate buffer in a decloaking chamber (121°C × 30 seconds, 90°C × 10 seconds) and washed with Tris buffer. Peroxide blocking was performed at ambient temperature for 30 minutes with 3% H_2O_2 in methanol. Protein blocking was performed with Dako serum-free protein block for 7 minutes. The slides were incubated with primary antibody at ambient temperature for 65 minutes and washed with Tris buffer, followed by incubation with Envision Dual-Link system-horseradish peroxidase (Dako) for 30 minutes. Staining was developed with 0.5% 3,3′-diaminobenzidine, freshly prepared with imidazole-HCl buffer, pH 7.5, containing hydrogen peroxide, and an antimicrobial agent (Dako) for 5 minutes and then counterstained with hematoxylin, dehydrated, and mounted.

The nuclear immunostaining (Fig. 1A) for both markers were quantified jointly by 2 pathologists (PY and LS) using a 4-value intensity score (0, 1+, 2+, and 3+) and the percentage (0–100%) of the extent of reactivity in each core. The final score was then obtained by multiplying the intensity and extension values (range, 0–300) as previously reported (20, 21).

Statistical analysis

Chi-square test or the Fisher exact test was used to determine category variable differences, and the Wilcoxon rank-sum test or Kruskal–Wallis test was used to detect continuous variable differences between groups. RFS and OS distributions were estimated using the Kaplan–Meier method. The log-rank test was used to determine survival

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Table 1. Summary of the clinicopathological characteristics of the squamous cell carcinoma and adenocarcinoma cases examined for EZH2 expression

Features	Categories	Squamous cell carcinoma ($N = 221$) *N* (%)	Adenocarcinoma ($N = 320$) *N* (%)
Sex	Female	90 (40.7)	177 (55.3)
	Male	131 (59.3)	143 (44.7)
Race	Caucasian	204 (92.3)	288 (90.0)
	Others	17 (7.7)	32 (10.0)
Smoking status	Never	4 (1.8)	54 (16.9)
	Former	114 (51.6)	142 (44.4)
	Current	103 (46.6)	124 (38.8)
T stage (IASLC)	T1	64 (29.0)	124 (38.8)
	T2	114 (51.6)	157 (49.1)
	T3	33 (14.9)	32 (10.0)
	T4	10 (4.5)	7 (2.2)
N stage (IASLC)	N0	143 (64.7)	237 (74.1)
	N1	58 (26.2)	51 (15.9)
	N2	20 (9.1)	32 (10.0)
TNM stage (IASLC)	I	102 (46.2)	203 (63.4)
	II	80 (36.2)	74 (23.1)
	III	39 (17.6)	43 (13.4)
Adjuvant therapy	No	162 (73.4)	230 (71.9)
	Yes	59 (26.7)	90 (28.1)
Vital status	Alive	86 (38.9)	177 (55.3)
	Dead	135 (61.1)	143 (44.7)
Recurrence status	No	141 (63.8)	205 (64.1)
	Yes	80 (36.2)	115 (35.9)

Age median: SCC, 69.0 years; adenocarcinoma, 66.3 years.
Tumor size median: SCC, 3.5 cm; adenocarcinoma, 2.7 cm.

differences between groups. Regression analyses of survival data, based on the Cox proportional hazards model, were conducted on RFS and OS rates. RFS duration was defined as the time of surgery to recurrence or last contact. OS was defined as the time of surgery to death or last contact. Associations between protein expression and clinico-pathologic variables were calculated using the median of the markers' expression: EZH2, 125.0 in SCC and 41.7 in adenocarcinoma, and TTF-1,150.0 in adenocarcinoma. We used C-index, which provides the area under the ROC curve for censored data (22) to evaluate the predictive accuracy of the Cox regression models. A C-index of 0.5 indicates that outcomes are completely random, whereas a C-index of 1 indicates that the model is a perfect predictor. SAS version 9.1, R 2.80 and S-Plus version 8.0 were used to carry out the computations for all analyses.

Results

Correlation between EZH2 expressions and clinico-pathologic features of NSCLC tumors

We first examined the levels of EZH2 immunohistochemical expression in NSCLC by histology type. We found that SCCs had a significantly ($P < 0.0001$) higher expression of nuclear EZH2 expression (mean 124.9; median 120.0) than adenocarcinomas (mean 56.6; median 61.1; Fig. 1B).

The correlations between EZH2 expression and clinico-pathologic characteristics of tumors are shown in Table 2. In SCC, EZH2 expression correlated only with pathological lymph node metastasis status (*N*), and tumors without metastasis (N0) expressed significantly ($P = 0.0001$) lower levels than tumors with metastasis (N1/N2). In lung adenocarcinoma, we found multiple significant associations when the EZH2 expression was compared with clinico-pathological variables. The expression of EZH2 was significantly higher in adenocarcinoma tumors from younger patients ($P < 0.0001$) and ever smokers ($P < 0.0001$). EZH2 expression was significantly higher in T2/T3/T4 adenocarcinomas compared with T1 tumors ($P = 0.0.001$), and in more advanced TNM stage ($P = 0.024$). Also, EZH2 showed a trend to higher expression in larger adenocarcinoma tumors ($P = 0.064$). Adenocarcinomas with any solid histologic pattern (defined as >5%) expressed significantly ($P < 0.0001$) higher levels of EZH2 than tumors without or with minimal (<5%) solid histology. Moreover, in the subset of adenocarcinomas with any

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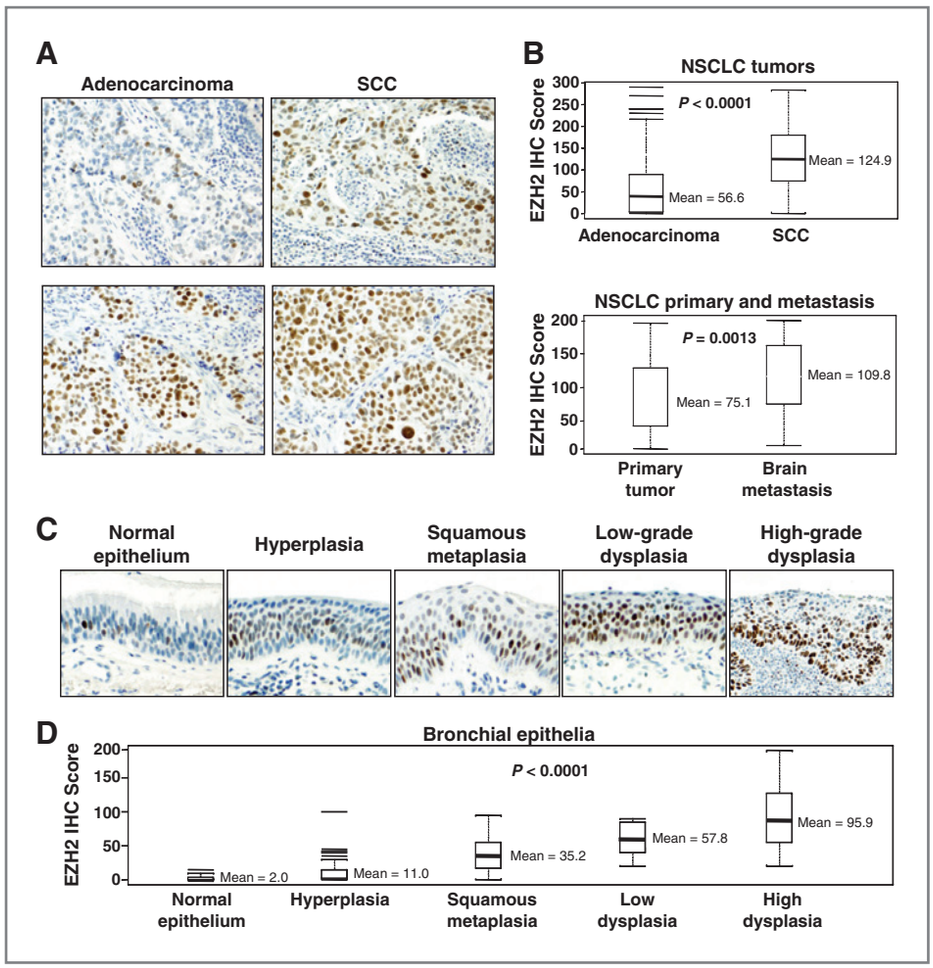

Figure 1. Representative photomicrographs of EZH2 nuclear immunohistochemical expression adenocarcinomas and squamous cell carcinomas of the lung (SCC; upper panels low expression, and lower panels high expression; ×200 magnification) (A). Box-plot showing EZH2 immunohistochemical expression of NSCLC tumors and primary NSCLC and corresponding brain metastasis (B). Representative photomicrographs of EZH2 nuclear immunohistochemical expression in bronchial epithelium with normal, mildly abnormal and preneoplastic histologies (×400 magnification) (C). Box-plot showing EZH2 immunohistochemical expression of bronchial epithelial samples (D).

solid pattern ($N = 125$), those tumors exhibiting higher percentage of solid component (greater or equal than the median of 40%) expressed significantly higher EZH2 ($P = 0.001$).

In adenocarcinomas, we examined the correlation between EZH2 protein expression and the mutation status of *KRAS* and *EGFR*, as well as the type of mutations detected. EZH2 expression was significantly lower in tumors with *EGFR* mutation ($P = 0.001$); however, this finding was not significant ($P = 0.165$) when we adjusted by smoking status. Although EZH2 expression did not correlate with the presence of *KRAS* mutation, we found that the expression levels were significantly higher in tumors with *KRAS* Gly to Cys substitutions ($P = 0.022$) compared with other amino acid changes (Gly to Ala, Val, Asp, Phe, and Ser).

EZH2 expression in advanced metastatic NSCLC tumors

To determine the expression of EZH2 in advanced metastatic NSCLC, we studied its expression in a series of 36 NSCLC primary tumors and corresponding brain metastasis. We found that EZH2 expression was significantly ($P = 0.0013$) higher in NSCLC brain metastases (mean 109.8; median 107.1) compared with primary tumors mean 75.1; median 75.1; Fig. 1B).

EZH2 expression in lung preneoplastic lesions

To investigate the potential role of EZH2 expression in the early pathogenesis of lung cancer, we examined its expression in a series of FFPE bronchial tissue samples with normal histology, and mildly abnormal and squamous dysplastic lesions obtained from patients with NSCLC. We focused on squamous preneoplastic lesion considering our finding that lung SCC demonstrated significantly higher expression than adenocarcinomas. We found a significant ($P < 0.0001$) increase in the expression of EZH2 with increasing severity of preneoplastic lesions, with high-grade dysplasias having the highest level of expression (mean 95.9, median 85.0) and normal epithelia (mean 2.0; median 0) and hyperplastic lesions (mean 11.0; median 7.0) having the lowest expression (Fig. 1C and D). Low-grade dysplasias (mean 57.8; median 45.0) and squamous metaplasias (mean 35.2; median 25.0) showed intermediate levels of EZH2 expression.

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Table 2. Summary of the correlations of EZH2 and TTF-1 protein expression and clinicopathological and molecular features of lung adenocarcinomas ($N = 320$)

			EZH2			TITF-1		
Features	**Categories**	**Cases *N* (%)**	**Mean**	**Median**	***P***	**Mean**	**Median**	***P***
Age	< Median[a]	160	71.10	61.50	<0.0001	132.58	160.00	0.964
	≥ Median[a]	160	42.06	20.00		128.96	144.00	
Sex	Male	143 (55.3)	59.09	50.00	0.336	114.29	135.00	0.002
	Female	177 (44.7)	54.56	30.00		144.09	160.00	
Smoking status	Never	54 (16.9)	23.14	0.00	<0.0001	147.10	160.00	0.162
	Ever	266 (83.11)	63.37	50.00		127.46	148.50	
Tumor size (average)	< Mean[b]	162 (62.2)	49.04	27.50	0.064	146.48	160.00	0.001
	≥ Mean[b]	158 (37.8)	64.31	50.00		114.68	125.00	
TNM	I	203 (63.5)	51.23	23.33	0.024	141.42	153.00	0.019
	II	74 (23.1)	66.00	58.33		108.24	112.00	
	III	43 (13.4)	65.64	60.00		119.32	143.00	
Histology pattern	No-solid	195 (60.9)	39.93	16.67	<0.0001	140.95	160.00	0.015
	Any solid (>5%)	125 (40.1)	82.56	73.33		114.90	123.00	
	Solid <40%	70	70.78	60.00	0.001	129.8	143.00	0.013
	Solid ≥40%	55	97.55	86.67		95.92	100.00	
EGFR mutation	Negative	252 (89.0)	60.60	46.67	0.001[c]	121.64	140.00	0.007
	Positive	33 (11.0)	29.90	3.33		167.45	180.00	
KRAS mutation	Negative	226 (76.9)	57.20	40.00	0.768	125.00	160.00	0.375
	Positive	68 (23.1)	51.96	43.33		134.61	140.00	
	Cys substitutions	32	58.38	60.00	0.022	162.40	180.00	0.005
	Other substitutions	36	46.25	21.60		109.91	126.50	

[a]66.3 years.
[b]2.7 cm.
[c]No significant ($P = 0.165$) after adjusting by smoking.

Association between EZH2 expressions and outcome in NSCLC patients

In SCC, no correlation between EZH2 expression and patients' OS and RFS was found (data not shown). Interestingly, in adenocarcinoma, the expression of EZH2 correlated significantly with patients' outcome. In univariate analysis, patients with adenocarcinoma showing higher than the median EZH2 expression (score = 41.7) had significantly lower OS [$P = 0.0001$; HR = 1.943; 95% confidence interval (CI), 1.387–2.723] and RFS ($P = 0.0014$; HR = 1.827; 95% CI, 1.261–2.648) rates compared with patients whose tumors expressed lower than the median score (Fig. 2A; and Supplemental Fig. S1A). These findings were confirmed in a multivariate analysis, after adjusting for clinical and pathological features (Table 3).

Association between combined EZH2 and TTF-1 expression and outcome in lung adenocarcinoma patients

As we (20, 23) and others (24–26) have published, TTF-1 protein expression is an important prognostic marker in patients with surgically resected lung adenocarcinomas. Therefore, we investigated the relevance of combined expression of EZH2 and TTF-1 with patient and disease characteristics. In adenocarcinomas, higher TTF-1 expression significantly correlated with female sex ($P = 0.002$), smaller tumor size ($P = 0.001$), and lower TNM stage ($P = 0.02$; Table 2). Consistently with our recent published data (20), TTF-1 expression level was significantly higher in lung adenocarcinomas with no-solid histology, and tumors with ≥40% solid pattern exhibited the lowest TTF-1 expression levels (Table 2).

As expected, tumors with high expression of TTF-1 (≥150 median score) correlated with better OS ($P = 0.0062$; HR = 0.624; 95% CI, 0.446–0.875) and RFS ($P = 0.0354$; HR = 0.674; 95% CI, 0.467–0.973) rates than patients whose tumors expressed low level of TTF-1. The multivariate analysis including the expression levels of EZH2 and TTF-1, after adjusting by age, sex, tumor size, TNM stage, and adjuvant therapy, showed that high EZH2 expression levels correlated significantly with worse OS and RFS rates, whereas high TTF-1 expression levels correlated significantly with better OS rates (Table 3).

To further fine-tune the ability of EZH2/TTF-1 to prognosticate lung adenocarcinomas, we defined 4 groups of patients according to the combined expression levels of EZH2 and TTF-1, using the same cut-offs that were utilized for the outcome analysis of each marker separately. The frequency of tumors in each group is the following:

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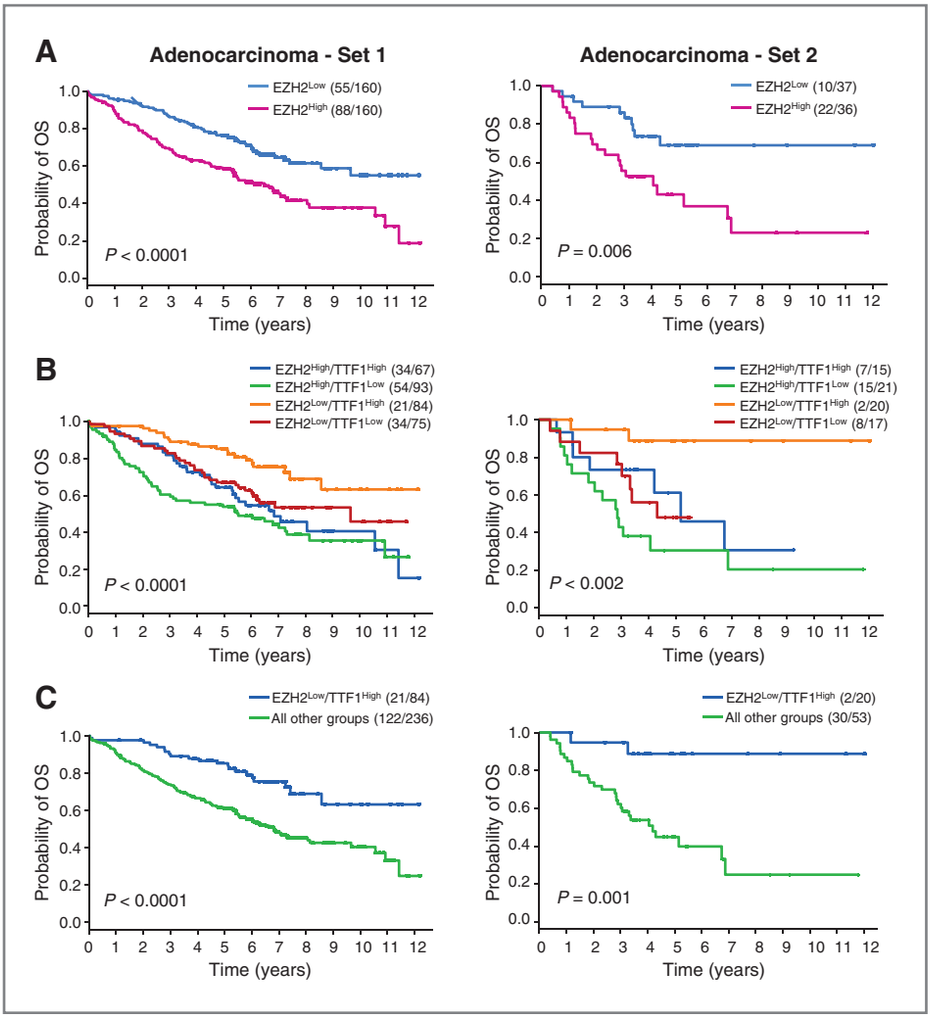

Figure 2. Kaplan–Meier curves depicting overall survival rates in the 2 sets of lung adenocarcinoma patients by EZH2 (A) and combined EZH2/TTF-1 expressions (B and C). In parenthesis, event/total number of cases. Set 1 (training), $N = 320$ tumors; Set 2 (validation), $N = 91$ tumors.

$EZH2^{Low}/TTF\text{-}1^{High}$, $N = 84$, 26%; $EZH2^{High}/TTF\text{-}1^{Low}$, $N = 67$, 21%; $EZH2^{High}/TTF\text{-}1^{High}$, $N = 93$, 29%; and $EZH2^{Low}/TTF\text{-}1^{Low}$, $N = 76$, 24%.

We found that patients with tumors expressing $EZH2^{Low}/TTF\text{-}1^{High}$ had a significantly better OS (Fig. 2B and C) and RFS (Supplemental Fig. S1B and S1C) rates than those patients from the other 3 groups, in both univariate and multivariate analyses (Supplementary Table S1 and Table 4).

Tumors expressing $EZH2^{Low}/TTF\text{-}1^{High}$ were more frequent in females ($P = 0.0005$), never smokers ($P = 0.008$), tumors of smaller size ($P = 0.016$), lower TNM stage ($P = 0.036$), and have more frequent *EGFR* mutations ($P = 0.003$). The correlation between $EZH2^{Low}/TTF\text{-}1^{High}$ and *EGFR* mutation remained significant ($P = 0.002$) when we adjusted by smoking status.

Validation analysis of the better outcome of patients with lung adenocarcinoma with $EZH2^{Low}/TTF\text{-}1^{High}$ expression

To validate our findings of better outcome in patients with $EZH2^{Low}/TTF\text{-}1^{High}$ expression, we studied a second cohort of 91 surgically resected adenocarcinoma tumors. In univariate analysis, we confirmed that patients whose tumors expressed higher than the median EZH2 score levels (score = 41.7) had statistically significant lower OS ($P = 0.0086$; HR = 2.736; 95% CI, 2.292–5.794) and RFS ($P = 0.0189$; HR = 2.425; 95% CI, 1.158–5.081) than patients whose tumors expressed lower than the median EZH2 levels (Fig. 2A; Supplemental Fig. S1A). However, these correlations were not significant on multivariate analyses.

Importantly, in univariate analysis, we confirmed that patients with tumors expressing $EZH2^{Low}/TTF\text{-}1^{High}$ had significantly better OS and RFS than those from the other 3 groups combined (Fig. 2B and C; Supplemental Fig. S1B and S1C). Multivariate analysis (Table 4) also demonstrated significantly better OS rates for patients with tumors having $EZH2^{Low}/TTF\text{-}1^{High}$ expression compared with the other groups combined, and a trend toward statistically significance difference for RFS rates.

We used C-index to evaluate the predictive accuracy of the Cox regression models. When the C-index was calculated for RFS and OS, we found that the clinical covariates

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Table 3. Summary of multivariate analysis of outcome in lung adenocarcinoma patients by expression of EZH2 and TTF-1

Features	Categories	*P* value	HR (95% CI)	Overall *P*[a]
Overall survival				
EZH2	Median (score 41.7)	0.0002	1.962 (1.383–2.782)	
TTF-1	Median (score 150.0)	0.0061	0.613 (0.433–0.870)	
Age	Median (66.3 years)	<0.0001	2.347 (1.644–3.351)	
Sex	Male vs. Female	0.0820	1.348 (0.963–1.887)	
Tumor size	Median (2.7 cm)	0.0301	1.467 (1.038–2.075)	
TNM stage	III vs. I	<0.0001	3.282 (1.987–5420)	<0.0001
	II vs. I	0.0379	1.537 (1.024–2.308)	
Adjuvant therapy	Yes vs. No	0.0169	0.583 (0.375–0.908)	
Recurrence-free survival				
EZH2	Median (score 41.7)	0.0249	1.542 (1.056–2.245)	
TTF-1	Median (score 150.0)	0.2289	0.793 (0.543–1.157)	
Tumor size	Median (2.7 cm)	0.0445	1.478 (1.01–2.164)	
TNM stage	III vs. I	<0.0001	4.647 (2.746–7.863)	<0.0001
	II vs. I	0.0021	2.022 (1.29–3.17)	
Adjuvant therapy	Yes vs. No	0.2885	0.789 (0.509–1.222)	

[a]*P* value for overall effect.

contained more predictive information than the markers (combined expression of EZH2/TTF-1) alone (Supplementary Table S2). In the lung adenocarcinoma testing set ($N = 320$), the combined model including both the clinical covariates and the combined expression of EZH2/TTF-1 led to the highest C-index: 0.70 for RFS and 0.69 for OS (Supplemental Table S2). The C-index was significantly different from 0.5 ($P < 0.001$), which indicated that the marker alone model is a significant predictor of RFS and OS. Similar C-index for the marker alone models for RFS (0.65) and OS (0.71) were also found in the validating dataset ($N = 91$).

Table 4. Summary of multivariate analysis of outcome of the 2 lung adenocarcinoma patients' cohorts by combined expression of $EZH2^{Low}$/$TTF\text{-}1^{High}$ versus other groups

		Cohort 1 ($n = 320$)			Cohort 2 ($n = 91$)		
Features	Categories	*P* value	HR (95% CI)	Overall *P*[a]	*P* value	HR (95% CI)	Overall *P*[a]
Overall survival analysis							
EZH2/TTF-1	$EZH2^{Low}$/$TTF\text{-}1^{High}$ vs. Others	0.0011	0.45 (0.278–0.726)		0.0466	0.221 (0.050–0.971)	
Age	Median (66.3 yrs)	<0.0001	2.058 (1.455–2.913)		0.6288	0.814 (0.354–1.873)	
Sex	Male vs. Female	0.1319	1.300 (0.924–1.828)		0.0478	2.226 (1.008–4.919)	
Tumor size	Median (2.7 cm)	0.0217	1.502 (1.061–2.126)		0.2213	1.672 (0.734–3.812)	
TNM stage	III vs. I	<0.0001	3.621 (2.166–6.054)	<0.0001	0.0016	4.768 (1.805–12.596)	0.0068
	II vs. I	0.0122	1.681 (1.120–2.523)		0.1834	1.825 (0.752–4.426)	
Adjuvant therapy	Yes vs. No	0.0097	0.549 (0.348–0.865)		0.3197	0.692 (0.252–1.568)	
Recurrence-free survival analysis							
EZH2/TTF-1	$EZH2^{Low}$/$TTF\text{-}1^{High}$ vs. Others	0.0087	0.51 (0.309–0.844)		0.0694	0.362 (0.121–1.084)	
Tumor size	Median (2.7 cm)	0.0639	1.435 (0.979–2.103)		0.1241	1.915 (0.837–4.384)	
TNM stage	III vs. I	<0.0001	4.671 (2.734–7.982)	<0.0001	0.0493	2.641 (1.003–6.954)	0.1445
	II vs. I	0.0011	2.110 (1.349–3.300)		0.7678	1.151 (0.452–2.932)	
Adjuvant therapy	Yes vs. No	0.2957	0.788 (0.505–1.231)		0.4047	0.678 (0.272–1.690)	

[a]*P* value for overall effect.

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Discussion

In this study, we investigated the clinical relevance of EZH2 protein expression in a large ($N = 541$) series of surgically resected NSCLCs, including 221 SCCs and 320 adenocarcinomas.

We determined that EZH2 expression is significantly higher in SCC compared with adenocarcinoma, and showed for the first time that NSCLC brain metastasis had significantly higher expression than corresponding primary lung tumors. Interestingly, EZH2 expression significantly increased with increasing severity of bronchial preneoplastic lesions. In lung adenocarcinoma, higher EZH2 expression significantly correlated with several clinico-pathological features, including younger patient age, smoking history, and higher TNM stage. In *KRAS* mutant adenocarcinomas, EZH2 expression correlated with the specific type of aminoacid substitution. Importantly, our analysis of a large number of cases of the 2 major types of NSCLC, demonstrated for the first time that higher EZH2 expression significantly correlated with worse outcome in patients with lung adenocarcinoma, but not in patients with SCC. In our series of lung adenocarcinomas, we identified a subset (26%) of tumors with low EZH2 and high TTF-1 expression that compared with all other tumors demonstrated better outcome in multivariate analysis for RFS and OS. These findings were confirmed in the analysis of a validation set of 91 lung adenocarcinomas. We conclude that EZH2 is highly expressed in NSCLCs, and its expression correlates with potential progression of preneoplastic lesions and development of metastasis. Importantly, EZH2 expression, particularly when combined with TTF-1 expression, is a prognostic marker in patients with surgically resected lung adenocarcinomas.

In NSCLC, previous studies have addressed the expression of EZH2 protein by immunohistochemistry (IHC) in archival tumor tissues of surgically resected tumors with controversial results (13, 14, 17, 27). Three of those studies examined the major histologic types, SCC and adenocarcinoma (13, 14). One of those studies included stage I to IV tumors and showed that SCCs ($N = 63$) expressed significantly higher EZH2 levels than adenocarcinomas ($N = 82$; ref. 13). In another study of NSCLCs, which included only stage I tumors, such difference was not detected (14). The third study of a combined series of 292 SCCs and adenocarcinomas showed that EZH2 expression did not correlate with patients' overall survival (27). The main limitation of those 3 studies is that they combined NSCLC histologies for the characterization of expression of EZH2 in tumors. Recent data have shown that significant differences exist in the molecular make-up of adenocarcinomas and SCCs of the lung (28), indicating the importance of characterizing molecular abnormalities in those tumor types separately. Recently, a third study using a very limited number ($N = 69$) of stage I to III surgically resected lung adenocarcinomas, reported that the expression of EZH2 by IHC was significantly higher in malignant cells than the corresponding adjacent normal lung tissues (17).

Our study represents the largest analysis of EZH2 protein expression in NSCLCs, and demonstrated for the first time that in lung adenocarcinomas high expression of this protein correlated with more aggressive tumor behavior, younger patient age, positive smoking history, and higher TNM stage. Our finding that in *KRAS* mutant lung adenocarcinomas, the expression of EZH2 correlates with the type of specific *KRAS* aminoacid substitution provides additional support to the notion that these lung tumors represent a molecularly heterogeneous group with different mechanisms of signaling occurring through mutant *KRAS* Gly to Cys compared with other aminoacid substitutions (29). Consistently with our preliminary studies (30), we found that EZH2 expression was higher in lung adenocarcinomas with less differentiated histology, reflected by a higher percentage of solid histology pattern. The association between overexpression of EZH2 and more aggressive tumor characteristics has been described in other solid tumor types, including, among others, prostate (8), breast (7), colon (11), and gastric (9) cancers. In addition, we have shown for the first time that EZH2 protein expression is significantly higher in NSCLC brain metastases compared with corresponding primary tumors suggesting that EZH2 overexpression may play a role in tumor progression and metastasis.

There is limited information available about the potential role of EZH2 in pathogenesis and progression of lung cancer. There is one study indicating that EZH2 protein expression was detected in tissues from premalignant dysplastic lesions of the lung (31), and that exposure to tobacco smoke condensate induced increased EZH2 expression in cultured lung cancer cell lines (32). We have shown for the first time that EZH2 expression increases with the increasing severity of preneoplastic lesions with squamous dysplastic features suggesting that abnormal expression of this protein may play a role in the early pathogenesis of lung cancer. These observations suggest that proteins of the polycomb repressive complex 2 (PRC2) as epigenetic gene modulators and are involved in tumor development. Their oncogenic function might be associated with their role in stem cell maintenance. In embryonic stem cells, PRC2-mediated epigenetic silencing maintains the pluripotent stem cell identity. This property may be harnessed during tumorigenesis. PRC2 proteins may thus play a central role in regulating stem cells, carcinogenesis, metastases, treatment resistance, and cancer survival by epigenetic silencing of key target genes.

One of most important findings of our study is that the expression of EZH2 alone and combined with the expression of TTF-1 significantly correlated with RFS and OS in patients with stage I to III surgically resected lung adenocarcinomas. We found that high levels of EZH2 protein were associated with worse RFS and OS after adjusting for other variables frequently associated with prognosis of surgically resected lung cancers, including age, sex, tumor size, TNM stage, and adjuvant therapy. These data are consistent with 2 previous studies that showed similar associations for overall survival in stage I surgically resected NSCLCs, mostly composed of adenocarcinoma and squamous cell carcinoma histologies (13, 14). Our IHC data in lung SCCs (not shown) does not demonstrate that high EZH2 is necessarily a bad prognostic marker.

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Higher levels of EZH2 protein expression assessed by IHC has been correlated with outcome in other solid tumors, including breast (7), colon (11), gastric (9), and ovary (12) cancers. The role of EZH2 in cancer development and progression coupled with the frequent high levels of expression of this protein in tumors has made this protein an interesting novel target in human cancers (5). Most of the preclinical work on the inhibition of EZH2 activity has used the carboxylic adenosine analog 3-deazaneplanocin (DZNep; ref. 33). DZNep has shown to inhibit cancer cell proliferation *in vitro*, in breast (34), ovary (35), prostate (34), and lung tumors (36), and its effect upon histone methylation my not be EZH2 specific (37). Interestingly, recent studies have shown that EZH2 expression contributed to resistance of ovarian cancer cells to cisplatin (35). In addition, and more recently, it was shown that miR-101 enhanced paclitaxel-induced apoptosis in NSCLC cell lines by direct repression of EZH2 (36).

As we (20, 23) and others (24–26) have reported high levels of IHC expression of TTF-1 protein correlated with improved patient outcomes. Based on the inverse correlation detected between the expressions of EZH2 and TTF-1 and with clinico-pathological features of lung adenocarcinoma, and particularly, with the opposite effect in patients' outcome, we investigated the prognostic effect of the combined expression of both markers defined by the median expression scores, we devised 4 groups of adenocarcinoma tumors. As expected, we found that patients with $EZH2^{Low}$/$TTF\text{-}1^{High}$ expression had a significantly better OS and RFS than those patients on the other 3 groups, in both univariate and multivariate analyses. These interesting observations were confirmed in a second cohort (validation cohort) of lung adenocarcinomas exhibiting similar clinico-pathological features than the first cohort (training cohort). Tumors with $EZH2^{Low}$/$TTF\text{-}1^{High}$ expression represented 26% of the adenocarcinomas examined, and expectedly, they correlated with clinico-pathologic features known to be associated with less aggressive lung adenocarcinoma behavior, including female sex, never smoking, smaller tumor size, lower TNM stage, and higher frequency of *EGFR* mutations after adjusting by smoking status.

One could speculate that there are two fundamentally distinct modes by which the carcinogenetic process can be initiated. The uncontrolled proliferation of pluripotent cells capable of self-renewal could be one such modality. The methylation of tumor suppressor genes could lead to an uncontrolled proliferation of stem cells with both pluripotent and self-renewing capabilities. The second modality is to reactivate the genes that are necessary for embryonic organogenesis. The TTF-1 gene is one such gene that is active during pulmonary organogenesis and once the organ is formed it is turned off to be reactivated again during carcinogenesis. Arguably tumors that are contextually driven in such a fashion have well-controlled proliferation and a more indolent phenotype with a longer natural history. In effect, that is what we see with TTF-1 positive tumors. It is also entirely plausible that the 2 mechanisms can coexist or interact, giving rise to the observed heterogeniety in tumor behavior. The mechanisms of this interaction are currently the topic of active investigation in our laboratory.

In summary, our findings indicate that high expression of EZH2 predicts for aggressive tumor behavior and correlates with more rapid progression of preneoplastic lesions to an invasive malignancy and development of metastasis. Importantly, EZH2 expression, particularly when combined with TTF-1 expression, allows us to more accurately predict the true prognosis of definitely treated adenocarcinomas of the lung.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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