

Prostaglandin E2 receptor 2 overexpression in squamous cell carcinoma of the lung correlates with p16^{INK4A} methylation and an unfavorable prognosis

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Abstract. Several studies have described p16^{INK4A} and prostaglandin E2 (PGE2) co-alterations in various solid tumors, including non-small cell lung cancer (NSCLC). In this study, we examined the correlation between PGE2 receptor 2 (EP2) expression and p16^{INK4A} methylation in NSCLC, and the association with clinicopathological features and prognostic significance. We retrospectively reviewed 88 NSCLC patients who underwent resection from July 1993 to May 1997. The tumors included 43 adenocarcinomas, 39 squamous cell carcinomas, and 6 large cell carcinomas. EP2 expression was determined by immunostaining, and p16^{INK4A} methylation was analyzed by methylation specific PCR. EP2 was overexpressed in 44% of NSCLC patients, 61% of adenocarcinoma cases, 28% of squamous cell carcinoma cases, and 33% of large cell carcinoma cases. EP2 expression positively correlated with lymph node metastasis ($P=0.034$), especially in patients with squamous cell carcinoma ($P<0.009$). Methylation of p16^{INK4A} was detected in 34% of NSCLC patients, 23% of adenocarcinoma cases, 44% of squamous cell carcinoma cases, and 50% of large cell carcinoma cases. In patients with squamous cell carcinoma, EP2 overexpression correlated with poor prognosis with a relative risk of 2.4 (confidence interval 2.1-51.8, $P<0.003$), and positively correlated with p16^{INK4A} methylation ($P<0.024$). Adenocarcinoma patients with p16^{INK4A} methylation had poor prognosis with a relative risk of 2.4 (confidence interval 1.8-69.7, $P<0.009$),

but this was not correlated with EP2 expression. In conclusion, EP2 overexpression was common in NSCLCs, especially in adenocarcinomas. Synchronous alteration of p16^{INK4A} and EP2 may accelerate progression of squamous cell carcinomas. These two alterations may differentially affect pathogenesis among subtypes of NSCLC.

Introduction

Lung cancer, particularly non-small cell lung cancer (NSCLC), is one of the most common cancers, and is also the leading cause of cancer death in western countries and in Japan (1,2). Currently, management of NSCLC is largely guided by tumor stage. Patients with early stage I and II tumors are treated by surgical resection either with or without adjuvant chemotherapy. Stage III patients require combined modality approaches that may include chemotherapy, radiation, and surgery. Nevertheless, the overall 5-year survival rates for these patients remain relatively poor, ranging from 70% for stage IA patients to 25% for stage IIIA patients whose tumors are surgically resectable (1). Most deaths are caused by metastatic recurrence. The differing survival outcomes among patients within a stage suggest the existence of other tumor factors that affect prognosis (3,4).

Prostaglandins (PG) are synthesized by the sequential action of phospholipases, cyclooxygenase 1 and 2 (COX1 and COX2), and specific terminal synthases. They exert their diverse biological effects through several membrane receptors. In particular, prostaglandin E2 (PGE2) is involved in various normal and pathological pathways that are mediated by four different E prostanoid receptors (EP1-4) (5). Differential expression of these EP receptors mediates the diverse and often antagonistic effects of PGE2 and its analogues on a variety of cell types (6,7). Furthermore, it has been reported that PGE2-EP3 signaling appears critical for tumor-associated angiogenesis, as well as tumor progression and tumor growth, in a mouse tumor implantation model (8). In human NSCLC, PGE2 may promote malignant growth by stimulating angiogenesis, tumor invasiveness, and apoptosis resistance, and by

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inhibiting immune surveillance (9-13). In addition, decreased level of PGE2 due to the use of celecoxib, a COX2 antagonist, was shown to inhibit cholangiocarcinoma cell growth by inhibition of cell cycle progression at the G₁-S checkpoint (14). Thus, the PGE2-EP pathways may play a pivotal role in the development of tumors.

DNA methylation plays an essential role in normal development and maintenance of genomic stability (15-17). However, alterations in methylation patterns frequently occur in tumor cells, and hypermethylation in the promoter regions of tumor suppressor genes (TSGs) is commonly associated with epigenetically mediated gene silencing in human cancer (18,19). One TSG, p16^{INK4A}, which is located on 9p21, encodes a cyclin-dependent kinase inhibitor that is important for G₁ cell cycle arrest (20,21). Promoter hypermethylation of this gene frequently occurs in human solid tumors (22). In lung cancer, p16^{INK4A} gene hypermethylation has been detected in 17% (23) to 84% (24) of cases in different studies.

Several studies have described the abnormal co-expression of p16^{INK4A} and PGE2 in various solid tumors, including NSCLC (25), breast cancer (26), and intrahepatic cholangiocarcinoma (27). However, there have been no reports on the correlation between p16^{INK4A} and EP2, which is one of the receptors of PGE2, in malignant tumors. In this study, we examined the correlation between EP2 expression and p16^{INK4A} methylation in NSCLC, as well as their association with clinicopathological features and the prognostic significance.

Patients and methods

Patients and tumors. We retrospectively reviewed data from 88 consecutive patients with NSCLC who underwent resection from July 1993 to May 1997 at the Chiba University Hospital in Chiba, Japan. Resected samples were immediately frozen and stored at -80°C until use.

Patients who had received chemotherapy or radiotherapy prior to resection were excluded from the study. The patients included 59 males and 29 females, with a mean age of 65 years (range 39-85 years) at diagnosis. Sixty-seven patients were smokers (including both current and former smokers), and 21 patients had never smoked. Patients were considered to have chronic obstructive pulmonary disease (COPD) if their post-bronchodilator FEV1/FVC ratio was <70% prior to operation (29). Staging was assessed using the TNM classification (28). Fifteen patients had stage IA disease, 16 patients had stage IB disease, 9 patients had stage IIB disease, 18 patients had stage IIIA disease, 21 patients had stage IIIB, and 9 patients had stage IV disease. Tumors were classified according to histopathologic type by two pathologists who performed all the pathologic examinations, and the chief pathologist who approved the final diagnosis. The histological subtypes included 43 adenocarcinomas, 39 squamous cell carcinomas, and 6 large cell carcinomas. This study was approved by the Institutional Review Board of Chiba University Hospital, and written informed consent was obtained from all participants.

p16^{INK4A} methylation assay. Genomic DNA was obtained from whole primary tumors after overnight digestion with sodium dodecyl sulfate and proteinase K (Life Technologies

Inc., Rockville, MD) at 37°C, followed by standard phenol-chloroform (1:1) extraction and ethanol precipitation. DNA was treated with sodium bisulfite as described previously (30). Treated DNA was purified with the Wizard DNA Purification System (Promega Corp., Madison, WI), desulfurated with 0.3 M NaOH, precipitated with ethanol, and resuspended in water. The p16^{INK4A} methylation status was determined by methylation-specific PCR (MSP), using primers specific for the methylated and unmethylated alleles. The primers for methylated p16^{INK4A} were TTA TTA GAG GGT GGG GCG GAT CGC (forward) and GAC CCC GAA CCG CGA CCG TAA (reverse), and the primers for unmethylated p16^{INK4A} were TTA GAG GGT GGG GTG GAT TGT (forward) and CAA CCC CAA ACC ACA ACC ATA A (reverse) (31). DNAs from 14 lymphocytes of healthy non-smoking volunteers were used as negative controls for the methylation-specific assays. As a positive control for the methylated alleles, the DNA from the lymphocytes of a healthy volunteer was treated with Sss1 methyltransferase (New England BioLabs, Inc., Beverly, MA) and then subjected to bisulfite treatment. Negative control samples without DNA were also included for each set of PCR. The PCR products were analyzed on 2% agarose gels containing ethidium bromide.

EP2 immunohistochemistry. Sections (5-μm thick) from formalin-fixed, paraffin-embedded biopsy specimens were deparaffinized in xylene and rehydrated. Antigen retrieval was performed using an autoclave. All sections were then immersed in 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The sections were incubated with primary antibodies for EP2 (Cayman Chemical Co., Ann Arbor, MI) at 4°C overnight. They were then treated with peroxidase using the labeled polymer method with Dako EnVision⁺ Peroxidase (Dako Corp., Carpinteria, CA) for 45 min. The peroxidase reaction was visualized using liquid 3,3'-diaminobenzidine substrate.

Semiquantitative analysis and staining interpretation. The staining intensity of EP2 expression was graded semiquantitatively into the following categories: none, weak, moderate, and strong. EP2 overexpression was considered positive if >30% of tumor cells were moderately or strongly stained. Slides were blindly evaluated at 3 different times, and the average levels were used for statistical analyses.

Statistical analysis. The correlations between p16^{INK4A} methylation status, EP2 expression, and clinicopathologic features were evaluated using either the Pearson chi-square test or the Fisher's exact test. Overall survival was analyzed with the Kaplan-Meier method, and differences in distribution were evaluated with the log-rank test. A Cox proportional hazards model was used for the multivariate survival analysis. Statistical differences were considered to be significant at the P<0.05 level. All data were analyzed using SPSS ver.12.0 (SPSS Inc., Chicago, IL).

Results

Association of p16^{INK4A} methylation status and EP2 with clinicopathologic features. Aberrant methylation of the

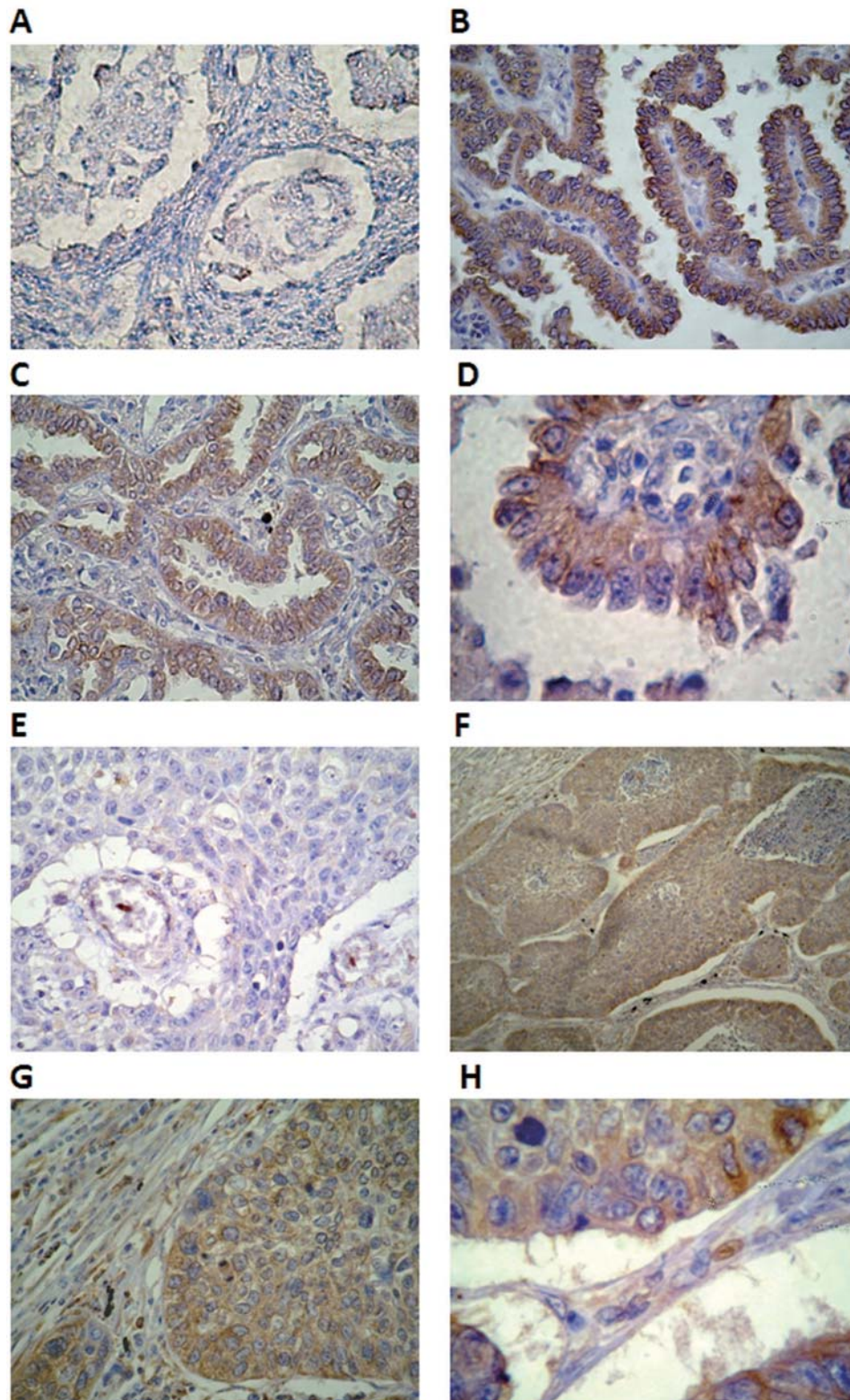


Figure 1. Immunostaining of EP2. EP2 expression in adenocarcinoma (A, negative at original magnification x40; B, positive at original magnification x10; C, positive at original magnification x40; and D, positive at original magnification x100). EP2 expression in squamous cell carcinoma (E, negative at original magnification x40; F, positive at original magnification x10; G, positive at original magnification x40; and H, positive at original magnification x100).

p16^{INK4A} gene was detected in 30 (34%) patients. It was methylated in 10 (23.3%) cases of adenocarcinoma, 17 (43.6%) cases of squamous cell carcinoma, and 3 (50%) cases of large cell carcinoma. There was no correlation between p16^{INK4A} methylation and clinicopathologic features.

EP2 was overexpressed in 39 (44%) patients (Fig. 1). It was frequently overexpressed in cases of adenocarcinoma (26 of 43; 60.5%), but less overexpressed in cases of squamous cell carcinoma (11 of 39; 28.2%) or large cell carcinoma (2 of 6; 33.3%) ($P < 0.01$). EP2 tended to be more frequently

Table I. Association of p16^{INK4A} methylation status and EP2 expression with clinicopathologic variables in patients with non-small cell lung carcinoma.

Variables	Total no. of patients	p16 ^{INK4A} No. of patients	%	P-value	EP2 No. of patients	%	P-value
Total	88	30	34.1		39	44.3	
Age, years							
≤66	46	14	30.4	0.449	22	47.8	0.488
>66	42	16	38.1		17	40.5	
Gender							
Male	59	22	37.3	0.367	22	37.3	0.058
Female	29	8	27.6		17	58.6	
Smoking history							
Non-smoker	21	5	23.8	0.255	13	61.9	0.063
Smoker	67	25	37.3		26	38.8	
COPD status							
Non-COPD	63	18	28.6	0.083	32	50.8	0.052
COPD	25	12	48		7	28	
Histology							
Adenocarcinoma	43	10	23.3	0.106	26	60.5	0.011
Squamous cell carcinoma	39	17	43.6		11	28.2	
Large cell carcinoma	6	3	50		2	33.3	
Differentiation							
Well-differentiated	33	11	33.3	0.908	14	42.4	0.782
Others	55	19	34.5		25	45.5	
Tumor stage							
Stage I and II	31	10	32.3	0.789	11	35.5	0.219
Stage III and IV	57	20	35.1		28	49.1	
Pathologic lymph node status							
Negative	48	15	31.3	0.137	18	37.5	0.034
Positive	40	15	37.5		21	52.5	
Adenocarcinoma							
Negative	21	3	14.3	0.174	11	52.4	0.289
Positive	22	7	31.7		15	68.2	
Squamous cell carcinoma							
Negative	23	10	43.5	0.987	7	30.4	0.009
Positive	16	7	43.8		5	31.3	

overexpressed in female, non-smokers, and non-COPD, although this was not statistically significant. EP2 significantly correlated with lymph node metastasis in all cases ($P < 0.034$), especially in patients with squamous cell carcinoma ($P < 0.009$, Table I). Tumor stage or differentiation did not have a significant effect on EP2 expression level.

We also examined the correlation between p16^{INK4A} methylation and EP2 expression. The frequency of p16^{INK4A}

methylation tended to be higher in cases of EP2 overexpression of all cases, although this was not significant. Interestingly, there was a positive correlation between p16^{INK4A} methylation and EP2 overexpression in cases of squamous cell carcinoma ($P < 0.024$, Fig. 2).

Survival analysis according to p16^{INK4A} methylation status and EP2 expression. In cases of squamous cell carcinoma,

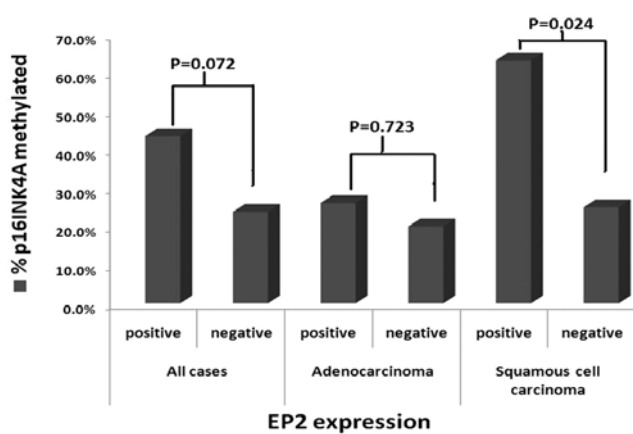


Figure 2. EP2 expression and correlation of p16^{INK4A} methylation for all cases of NSCLC, as well as cases of adenocarcinoma and squamous cell carcinoma. The frequency of p16^{INK4A} methylation was higher in the EP2 positive (63.2%) than the EP2 negative cases (25%) among squamous cell carcinoma cases (P=0.024).

patients with EP2 overexpression had significantly poorer prognosis (P<0.04, Table II). Even when controlling for other covariables, EP2 overexpression was an independent prognostic factor (relative risk = 2.4, confidence interval 2.1-51.8, P<0.003, Table III). On the other hand, adeno-

carcinoma patients with p16^{INK4A} methylation displayed significantly poorer prognosis than non-methylated cases (P<0.004, Table II and Fig. 3). Even when controlling for other covariables, p16^{INK4A} methylation was also an independent prognostic factor (relative risk = 2.4, confidence interval 1.8-69.7, P<0.009, Table III).

Discussion

PGE2 has been reported to promote malignant growth by stimulating angiogenesis, tumor invasiveness, apoptosis resistance, and by inhibiting immune surveillance in NSCLC (9-13). In addition, metastatic lymph nodes have been shown to express COX2 at higher levels than non-metastatic lymph nodes in breast cancer, and this was paralleled by higher PGE2 tissue levels (32). To our knowledge, this is the first study to report EP2 expression, one of the receptors of PGE2, in NSCLC and to examine its impact on survival. EP2 overexpression was more frequently found in patients with lymph node metastasis than in those without lymph node metastasis, especially in cases of squamous cell carcinoma. Those with squamous cell carcinoma and EP2 overexpression showed unfavorable prognosis. Thus, alteration of the COX2-PGE2-EP2 pathway may accelerate tumor invasion and metastasis in NSCLC, especially in squamous cell carcinoma.

Table II. Survival analysis according to p16^{INK4A} methylation status and EP2 expression.

Variable	No. of patients	Survival time, months	Confidence interval	P value
p16 ^{INK4A} methylation				
Unmethylated	58	91.4	(73.2- 109.6)	0.074
Methylated	30	60.1	(38.4-83.7)	
Adenocarcinoma				
Unmethylated	33	114	(91.4-136.6)	0.004
Methylated	10	32.5	(18.9-46.1)	
Squamous cell carcinoma				
Unmethylated	22	63.1	(39.4-86.9)	0.354
Methylated	17	89.1		
EP2 expression				
Negative	49	80.2	(62.0-98.5)	0.706
Positive	39	79.5	(56.8-102.2)	
Adenocarcinoma				
Negative	17	100.1	(68.8-131.3)	0.648
Positive	26	96	(68.5-123.5)	
Squamous cell carcinoma				
Negative	27	88.3	(64.8-111.9)	0.044
Positive	12	48.3	(11.9-84.7)	

Table III. Multivariate Cox regression analysis in 88 patients with non-small cell lung cancer.

Variable	All cases (n=88)		Adenocarcinoma (n=43)		Squamous cell carcinoma (n 39)	
	Relative risk (95% CI)	P-value	Relative risk (95% CI)	P-value	Relative risk (95% CI)	P-value
p16 ^{INK4A} methylation	0.4 (0.6-3.2)	0.377	2.4 (1.8-69.7)	0.009	0.7 (0.1-1.8)	0.286
EP2	0.02 (0.4-3.3)	0.962	0.2 (0.1-5.4)	0.816	2.3 (2.1-51.8)	0.003
Gender	0.4 (0.52-4.57)	0.437	1.4 (0.3-2.3)	0.222	0.5 (0.1-21)	0.677
Age	0.01 (0.95-1.03)	0.786	0.1 (0.8-1.1)	0.194	0.1 (0.9-1.2)	0.105
Smoking	0.7 (0.56-6.59)	0.29	1.7 (0.3-98.6)	0.271	0.2 (0.0-17.1)	0.888
COPD	0.3 (0.43-4.17)	0.614	0.9 (0.1-126)	0.67	1.8 (0.6-70.7)	0.132
FEV1	0.02 (0.99-1.05)	0.197	0.01 (0.96-1.1)	0.659	0.0 (0.9-1.1)	0.875
Histopathologic type	0.6 (0.24-1.34)	0.198				
Pathological stage	2.2 (0.02-0.53)	0.006	3.6 (0.0-1.2)	0.02	0.2 (0.1-5.6)	0.85
Lymph node metastasis	0.2 (0.44-3.11)	0.74	0.7 (0.0-9.5)	0.653	1.7 (0.9-38.5.4)	0.06

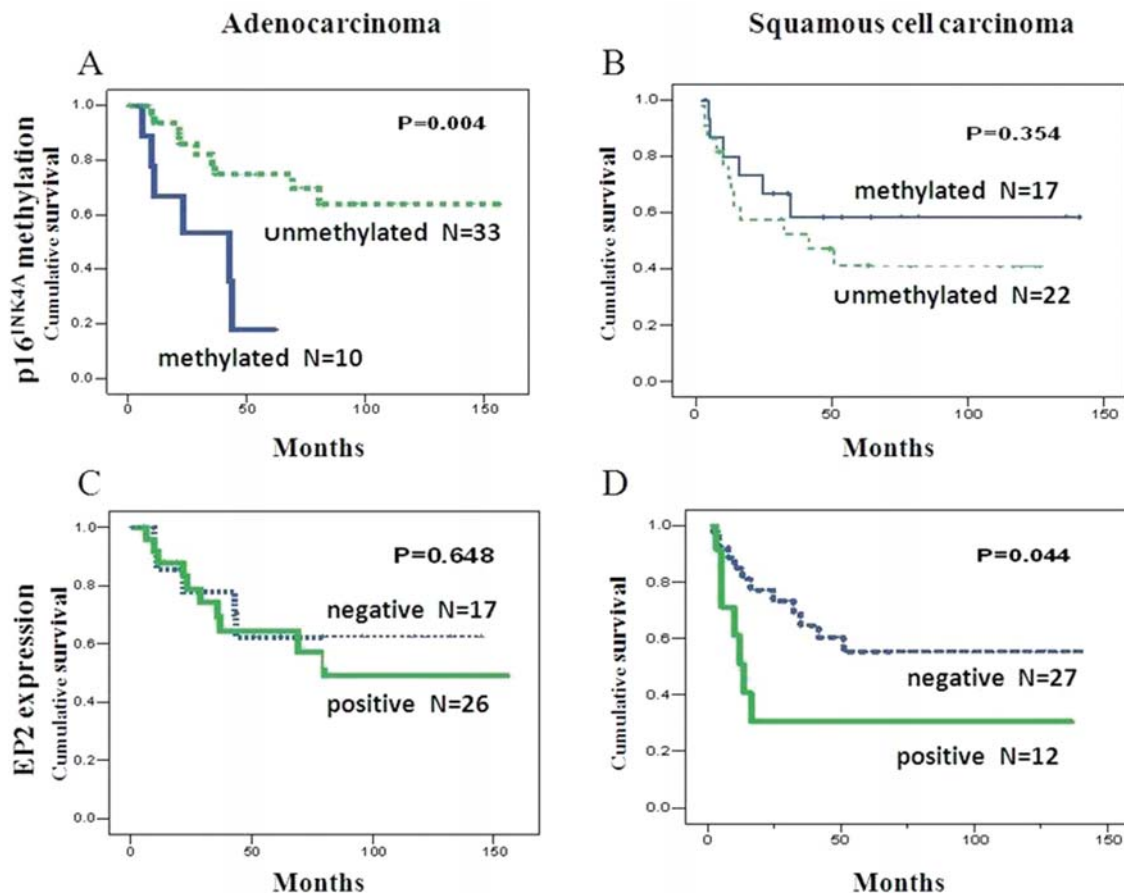


Figure 3. Kaplan Meier survival curves. A, adenocarcinoma patients with p16^{INK4A} methylation (N=10) had significantly poorer prognosis than unmethylated cases (N=33, P<0.004). B, there was no significant prognostic impact of p16^{INK4A} methylation in cases of squamous cell carcinoma. C, there was no significant prognostic impact of EP2 expression in cases of adenocarcinoma. D, squamous cell carcinoma patients with positive EP2 expression (N=12) had significantly poorer prognosis than EP2 negative cases (N=27, P<0.044).

Aberrant methylation of TSGs is a significant process in carcinogenesis. In particular, methylation of the p16^{INK4A} gene is accepted as being a crucial event in various malignancies,

including lung cancer (33-37). It has also been associated with a higher recurrence rate in stage I NSCLC (38). In our study, aberrant methylation of the p16^{INK4A} gene was significantly

related to unfavorable prognosis in lung adenocarcinomas. Similar findings have been previously reported (39,40), while others reported unfavorable prognosis in lung squamous cell carcinomas (41). Thus, aberrant methylation of the p16^{INK4A} gene may reveal a malignant potential in NSCLC. Further prospective study with a larger cohort is needed to confirm the prognostic value of p16^{INK4A} methylation in NSCLC.

Several studies have described the association of the p16^{INK4A} gene alteration and PGE2 or COX2 overexpression in various malignancies (25-27). It was reported that COX2 promotes human cholangiocarcinoma growth, and that this effect could be inhibited by celecoxib, a COX2 antagonist, through blockade of the cell cycle at the G₁-S checkpoint but not at the G₂-M transit (14). In addition, the p16^{INK4A} gene has been shown to act as a cyclin-dependent kinase inhibitor that is important for G₁ cell cycle arrest (20,21). Thus, p16^{INK4A} may be activated through the COX2-PGE2-EP2 pathway, and then play a role in cell cycle regulation at the G₁ phase. In our study, methylation of p16^{INK4A} was a poor prognostic factor in adenocarcinoma, while EP2 overexpression was a poor prognostic factor in squamous cell carcinoma. It seems that both alterations target the same cell cycle regulation, but the effects of dysregulation on the malignant potential are different between adenocarcinoma and squamous cell carcinoma.

Romanov *et al* (42) and Crawford *et al* (43) studied morphologically normal human mammary epithelial cells with p16^{INK4A} gene methylation, and found that late-stage serial cultures of these cells showed malignant transformation. They reported that the malignant potential of these cells was affected by three synergistically acting factors. First, the loss of the p16^{INK4A} protein relieves cyclin D1/cyclin-dependent kinase 4/6-control during G₁/S transition and allows the subsequent unrestrained phosphorylation of pRB by cdk4/6; thus, in the absence of these restraints and the presence of adequate nutrients and mitogenic factors, these cells have an extended replicative potential (42,44). Second, telomere dysfunction not only provides the genetic mutations required for cancer initiation (45), but also instigates an apoptotic response (46). Third, the COX2-induced inhibition of apoptosis and immune surveillance, and remodeling of the stroma, along with induction of angiogenesis, supports this malignant program. They postulated that the critical events in this process (loss of cell cycle checkpoint control, telomere dysfunction, DNA damage, and sustained induction of COX2) provide a mechanistic framework for malignant transformation. Thus both p16^{INK4A} and the COX2-PGE2-EP2 pathways have a role in malignant transformation. Along with these findings, the positive correlation between p16^{INK4A} methylation and EP2 expression in squamous cell carcinoma patients in this study should provide insight into tumor progression, warranting further evaluation of the EP2 and p16^{INK4A} methylation association, as well as their role in cell cycle regulation.

In conclusion, EP2 overexpression was common in NSCLCs, especially in adenocarcinoma. Methylation of p16^{INK4A} was a poor prognostic factor in adenocarcinoma patients. In cases of squamous cell carcinoma, EP2 overexpression correlated positively with lymph node metastasis and p16^{INK4A} methylation, and was a poor prognostic factor. Thus, these two alterations differentially affect the pathogenesis

of adenocarcinoma and squamous cell carcinoma. In addition, the synchronous alteration of p16^{INK4A} and EP2 in squamous cell carcinoma may provide novel biological insights into the mechanisms of squamous cell carcinoma.

Acknowledgements

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