The Korean Journal of Pathology 2010; 44: 469-76
DOI: 10.4132/KoreanJPathol.2010.44.5.469

Relationship between the Endogenous Hypoxic Markers Hypoxia Inducible Factor-1α, Carbonic Anhydrase IX, and Epithelial Mesenchymal Transition Regulator TWIST Expression in Non-small Cell Lung Cancer

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Received : March 3, 2010
Accepted : May 25, 2010

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*This work was supported by grant 2009032 from the Medical Research Institute, Pusan National University, Busan, Korea.

Background : The epithelial mesenchymal transition (EMT) is intimately associated with tumor hypoxia. The present study was conducted to investigate the immunohistochemical relationship between hypoxic and EMT-related molecules in non-small cell lung carcinoma (NSCLC). **Methods :** Immunohistochemical staining for hypoxia inducible factor (HIF)-1α, carbonic anhydrase (CA) IX, TWIST, and E-cadherin proteins was performed in 146 cases of NSCLC (80 cases of adenocarcinoma and 66 cases of squamous cell carcinoma) using tissue microarray blocks. **Results :** HIF-1α, TWIST, CA IX, and E-cadherin were expressed in 58 (40%), 90 (62%), 82 (56%), and 36 (25%) of 146 NSCLC cases, respectively. TWIST expression was positively correlated with HIF-1α expression ($p = 0.03$) and inversely correlated with E-cadherin expression ($p < 0.01$). TWIST and CA IX expression were not significantly interrelated, but each showed a relationship with histological tumor grade. However, the expression of these molecules had no significant effect on clinical staging or patient survival. **Conclusions :** Although TWIST expression was correlated positively with HIF-1α expression and inversely correlated with E-cadherin, HIF-1α expression was not associated with E-cadherin expression. However, considering the relationship between HIF-1α and TWIST expression, further studies should be performed to demonstrate the role of hypoxia-induced EMT in NSCLC.

Key Words : Carcinoma, non-small-cell lung; Epithelial mesenchymal transition; Immunohistochemistry

The epithelial mesenchymal transition (EMT) is an important process during embryonic morphogenesis in which epithelial cells lose their ability of cell-cell interaction and acquire a highly motile mesenchymal phenotype.[1,2] Recently, EMT has been recognized as a potential mechanism for carcinoma progression.[1-3] Cancer cells that undergo EMT fail to express E-cadherin (encoded by *CDH1*), which obviates cell-cell adhesion and leads to the acquisition of invasive and metastatic potential. Several transcription factors, including Snail, ZEB, and the basic helix-loop-helix (bHLH) families, which strongly repress *CDH1* transcription, induce EMT.[1-3] EMT is vital in various human tumors including breast,[4,5] ovarian,[6] and gastric cancers.[7] Among various EMT regulators, the bHLH family member TWIST is a master regulator of gastrulation and mesoderm specification during normal embryogenesis and plays an important role in tumor-related EMT.[8-12]

The tumor microenvironment also influences tumor progression and may be related to EMT and expression of Snail, bHLH factors, and ZEB.[9] Among the various known microenvironmental factors, hypoxia is emerging as a potentially important player in the induction of EMT.[13,14]

The role of EMT in non-small cell lung carcinoma (NSCLC)

has been reported.[15-17] An immunohistochemical study of EMT in human NSCLC implicated EMT as a poor prognostic factor.[16] Hypoxia has also been studied extensively in NSCLC and has been linked with a poor prognosis because of the induction of tumor angiogenesis by activating hypoxia inducible factor (HIF)-1α, which enhances tumor growth and metastasis.[18-20] Several studies have shown that TWIST actively participates in EMT during hypoxia.[21-24] These studies prompted us to investigate the relationship between hypoxia and TWIST expression in NSCLC.

HIF-1α is a transcriptional factor that is rapidly degraded in normoxic conditions but is stabilized under hypoxic conditions, and the carbonic anhydrase (CA) IX transmembrane protein is a well-known endogenous hypoxic marker.[20] CA IX correlates with HIF-1α expression and is also a poor prognostic factor in NSCLC.[20] The present study was conducted with the assumption that the expression of the endogenous hypoxic markers HIF-1α and CA IX would be correlated with TWIST expression and could lead to the loss of E-cadherin expression in NSCLC.

MATERIALS AND METHODS

Clinicopathological data of the patients with NSCLC

All patients with NSCLC who underwent a lobectomy or pneumonectomy at Pusan National University Hospital, Busan, Korea from June 1998 to October 2007 were enrolled. After excluding cases in which there were insufficient pathological materials remaining for further study, 146 cases (109 men and 37 women; mean age, 63.7 years; range, 40 to 85 years) were selected. Tumors were staged according to the tumor, node and metastasis classification of the International Union Against Cancer Staging System[25] after review of the clinical, radiological, and pathological data. Other clinical information was extracted from medical records.

There were 66 cases of squamous cell carcinoma (SqCC) and 80 cases of adenocarcinoma (AdC). Representative tumor areas to be sampled for tissue microarray (TMA) were carefully selected and marked on slides used for hematoxylin and eosin staining. A large-diameter stylet (2.0 mm) was used, considering tumor heterogeneity. The studied specimens were routinely oversampled with two replicate core tumor region samples (different areas) from each donor block. Forty-eight cores from 24 tumors were included in each tissue array block. Multiple 4 μm-thick sections were cut with a microtome.

Immunohistochemistry

Sections from TMA blocks were transferred to poly-L-lysine-coated glass slides and air-dried overnight at 37℃. The slides were dewaxed in xylene (three changes), rehydrated in a graded series of decreasing ethanol concentrations, and rinsed in pH 7.4 Tris-buffered saline (TBS). Endogenous peroxidase activity was inactivated with 5% hydrogen peroxide in methanol for 15 minutes at 37℃. Antigen retrieval for TWIST (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CA IX (Novus Biologicals, Littleton, CO, USA), and E-cadherin (DakoCytomation, Carpinteria, CA, USA) was performed using a 5 minute microwave treatment in TBS. For anti-HIF-1α (Novus Biologicals), slides were subjected to microwave irradiation three times for 5 minutes, and an anti-HIF-1α antibody (Novus Biologicals) was applied. Each antibody was incubated with tissue sections at room temperature for 1 hour. The Catalyzed Signal Amplification kit II (DakoCytomation) was used to detect the HIF-1α antibody signal, according to the manufacturer's instructions. The Envision Detection kit (DakoCytomation) was used for the other antibodies. Reaction products were visualized by exposing sections to diaminobenzidine. Nuclei were lightly counterstained for approximately 20 seconds with Mayer's hematoxylin. Grade 3 transitional cell carcinoma was used as a positive control for TWIST. A glioblastoma multiform specimen was used as a positive control for HIF-1α and CA IX, and infiltrating ductal carcinoma was used for E-cadherin. Each primary antibody was replaced with mouse IgG as a negative control.

Immunohistochemistry scoring

Only nuclear staining was considered for HIF-1α. Upon examination of the two TMA cores, the cases showing an average of > 10% tumor cell nuclei staining were considered positive. A two-way scoring system was used for the results analysis. For TWIST and CA IX, the staining intensity on each TMA spot was scored in four categories on a 0-3 scale: negative (0), weak (1), moderate (2), and strong (3). The proportion of positive cells of interest was then estimated in each sample using a 1-4 point scale: ≤ 25% (1), 25-50% (2), 50-75% (3), and > 75% (4). The average score for each sample was calculated by multiplying and dividing by four groups (0, 1-3, 4-6, and 9-12). A score of 0-3 was considered negative and one of 4-12 as positive. E-cadherin immunostaining was also divided into four categories: strong membranous staining in > 80% of cells (+++); homogeneous positive tumor cells (more than 50-80% positive tumor cells,

++); heterogeneous staining (20-50% positive tumor cells, +); weak staining (< 20% positive tumor cells, or no evidence of membrane staining). The 2+ and 3+ groups were defined as positive and the 1+ and weak staining group as negative.

Statistical analyses

The Pearson's chi-square test or Fisher's exact test were used to evaluate the statistical significance of the immunohistochemical results related to the clinicopathological parameters. Follow-up information was also obtained for survival analysis. Patient survival was calculated as the time between operation and death. Patients who were still alive at the time of data collection were censored in the statistical analysis. Survival curves were plotted according to the Kaplan-Meier method. Differences between the survival curves were analyzed using the log-rank test. All statistical analyses were performed with SPSS ver. 15.0 (SPSS Inc., Chicago, IL, USA). p-values < 0.05 were considered statistically significant. All statistical tests were two-sided.

RESULTS

HIF-1α, CA IX, TWIST, and E-cadherin expression

TWIST was not expressed in normal lung tissue (Fig. 1A), but it was expressed mainly in the cytoplasm or perinuclear area in 82 of 146 NSCLC cases (56%) (Fig. 1B). Nuclear TWIST staining was infrequently noted in 14 cases (9.5%) (Fig. 1C). HIF-1α stained dark brown in tumor cell nuclei in 58 cases (40%) (Fig. 2A), but it was not expressed in normal bronchial epithelia or alveolar pneumocytes. CA IX (Fig. 2B) and E-cadherin (Fig. 2H) stained mainly along the tumor cell membranes and infrequently in the cytoplasm. CA IX was positive in 90 cases (62%), and E-cadherin was positive in 36 cases (25%).

Relationship among HIF-1α, CA IX, TWIST, and E-cadherin expression and the clinicopathological factors

HIF-1α and CA IX expression were higher in SqCC than in AdC ($p < 0.01$ and $p = 0.01$, respectively). Cytoplasmic TWIST and CA IX expression increased with tumor grade ($p = 0.04$, $p =$

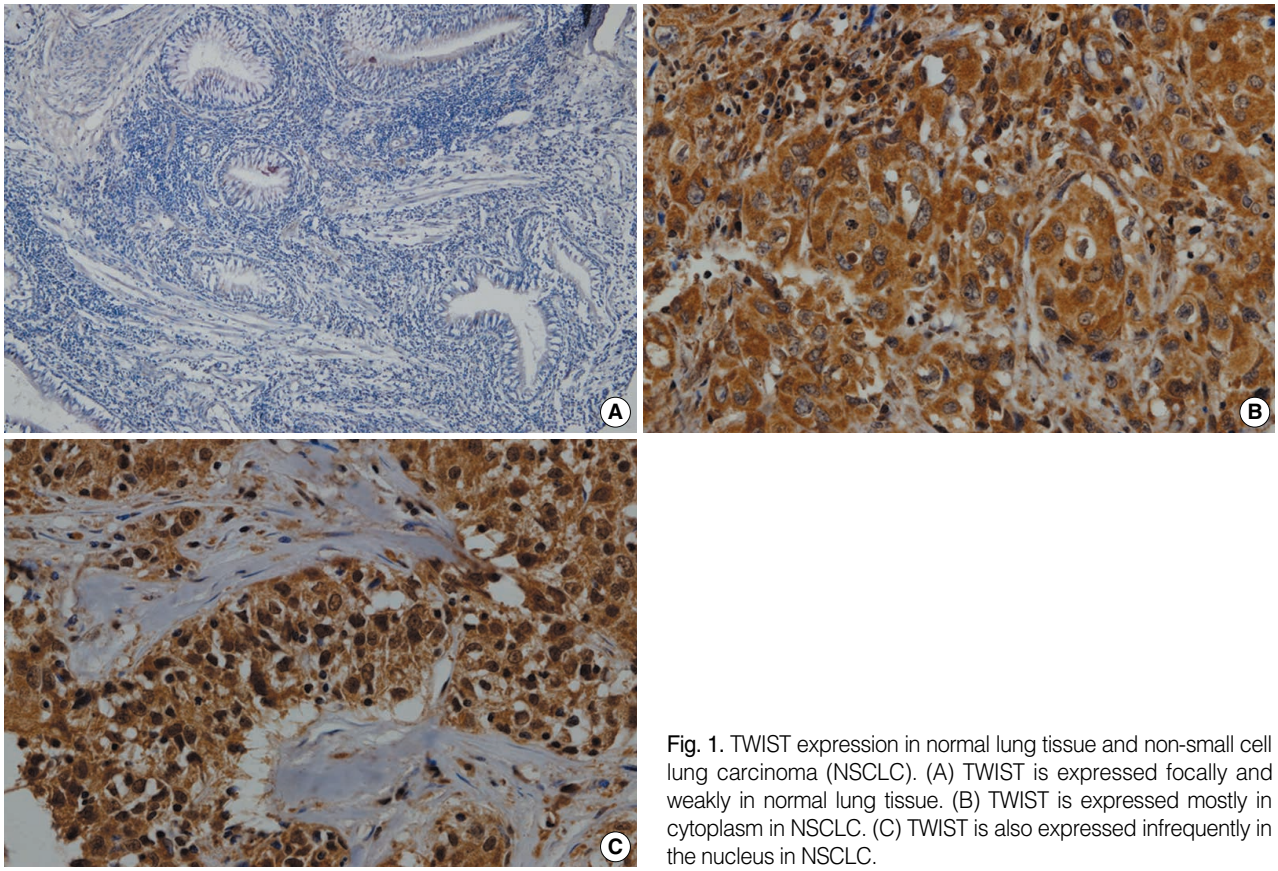

Fig. 1. TWIST expression in normal lung tissue and non-small cell lung carcinoma (NSCLC). (A) TWIST is expressed focally and weakly in normal lung tissue. (B) TWIST is expressed mostly in cytoplasm in NSCLC. (C) TWIST is also expressed infrequently in the nucleus in NSCLC.

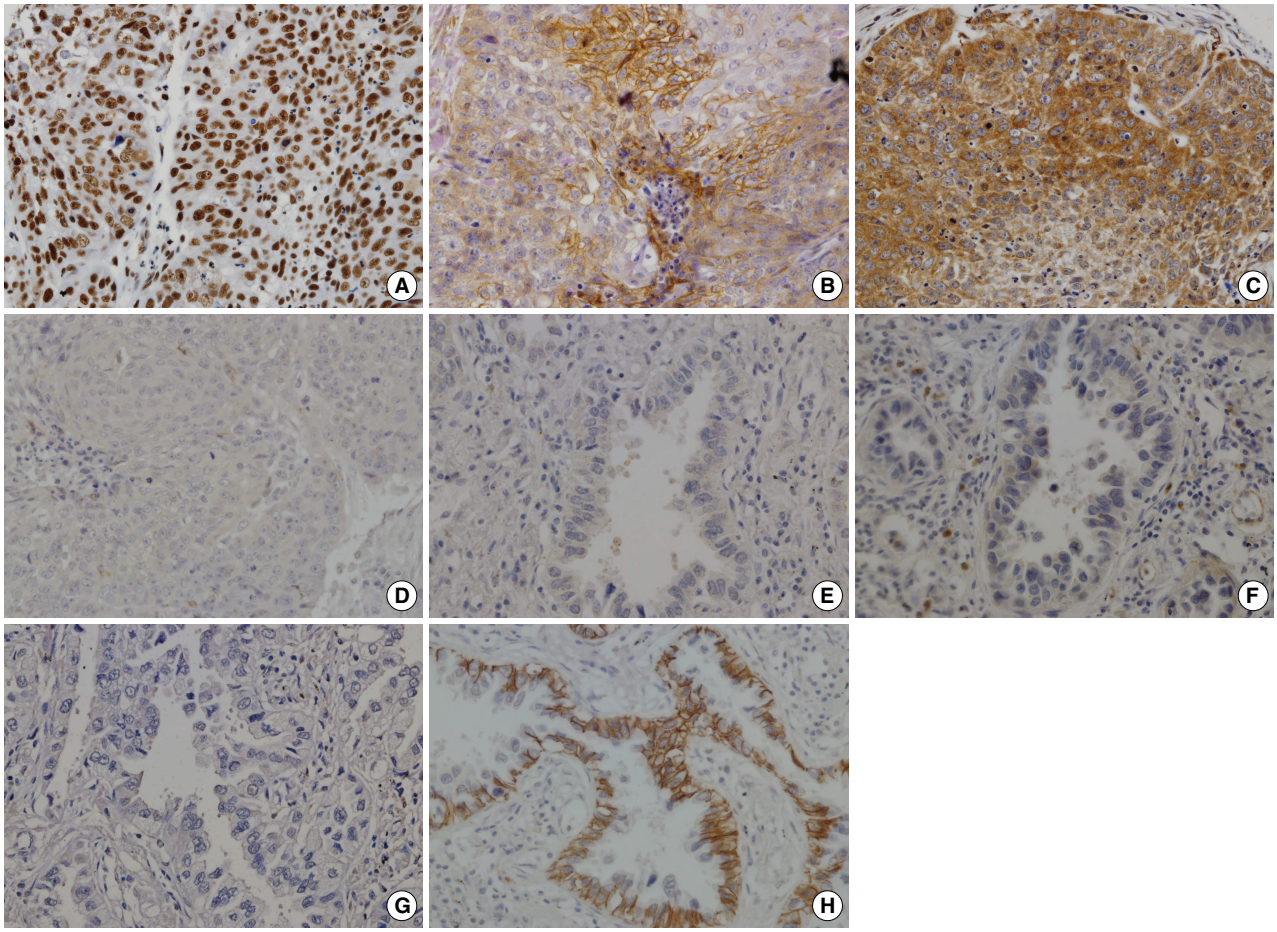

Fig. 2. Hypoxia inducible factor (HIF)-1α (A, E), carbonic anhydrase (CA) IX (B, F), TWIST (C, G), and E-cadherin (D, H) expression. In the panel (A-D) from the same tumor core, HIF-1α is strongly expressed in nuclei (A). CA IX (B) and TWIST (C) show simultaneous positive staining, whereas E-cadherin (D) expression is lost. In the panel (E-H) from the other tumor core, HIF-1α (E), CA IX (F), and TWIST (G) are negative, whereas E-cadherin is positive (H).

0.03, respectively) (Tables 1, 2). HIF-1α expression also showed a tendency to increase with higher tumor grade, but the difference was not significant. TWIST nuclear positivity was observed in 14 cases, but it failed to show a significant relationship with the clinicopathological factors.

Mutual TWIST, HIF-1α, CA IX, and E-cadherin expression

TWIST expression was positively correlated with HIF-1α expression ($p = 0.03$) and inversely correlated with E-cadherin expression ($p < 0.01$) (Table 3). TWIST and CA IX expression were not significantly associated (Table 3). Binary logistic regression was performed to further define the relative role of HIF-1α and CA IX in TWIST expression. HIF-1α expression was correlated with TWIST expression (Table 3). The odds ratio for TWIST expression in HIF-1α positive samples was 2.1 compared to that in the HIF-1α negative group (95% confidence interval, 1.06 to 4.29) (Fig. 3A), whereas CA IX expression was not related to TWIST expression. To investigate the effect of HIF-1α and TWIST expression on the loss of E-cadherin expression, patients were divided into four groups according to HIF-1α and TWIST expression: group 1, negative/negative; group 2, negative/positive; group 3, positive/negative; and group 4, positive/positive. The percentage of cases in which E-cadherin expression was lost was 18.5%, 25.3%, 8.2%, and 23.3% in groups 1, 2, 3, and 4, respectively (Fig. 3B). The loss of E-cadherin expression was related to the TWIST-positive group regardless of HIF-1α expression ($p = 0.02$). Other clinicopathological factors did not show a statistically significant difference according to group. HIF-1α and CA IX expression showed a weak but significant correlation ($r = 0.18$, $p = 0.03$).

Table 1. Relationship between hypoxia inducible factor (HIF)-1α and carbonic anhydrase (CA) IX expression with clinicopathological factors in patients with non-small cell lung carcinomas

Factors	HIF-1α expression		p-value	CA IX expression		p-value
	Negative	Positive		Negative	Positive	
Age (yr)						
≥ 64	47	38	0.20	28	33	0.11
< 64	44	20		28	57	
Sex						
Male	60	49	0.03	37	72	0.06
Female	28	9		19	18	
Histology						
SqCC	29	37	< 0.01	18	48	0.01
AdC	59	21		38	42	
Grade						
I	29	11	0.06	21	19	0.03
II, III	59	47		35	71	
pT						
I	32	16	0.26	25	23	0.02
II-IV	56	42		31	67	
pN						
0	62	42	0.79	36	68	0.14
I-III	26	16		20	22	
Stage						
I	59	36	0.73	34	61	0.53
II	10	9		7	12	
III	19	13		15	17	

SqCC, squamous cell carcinoma; AdC, adenocarcinoma.

Table 2. Relationship between TWIST and E-cadherin expression with clinicopathological factors in patients with non-small cell lung carcinomas

Factors	TWIST		p-value	E-cadherin		p-value
	Negative	Positive		Negative	Positive	
Age (yr)						
≥ 64	29	32	0.44	47	14	0.68
< 64	35	50		63	22	
Sex						
Male	47	62	0.77	84	25	0.47
Female	17	20		26	11	
Histology						
SqCC	29	37	0.98	55	11	0.04
AdC	35	45		55	25	
Grade						
I	23	17	0.04	29	11	0.62
II, III	41	65		81	25	
pT						
I	23	25	0.48	32	16	0.25
II-IV	41	57		78	20	
pN						
0	45	59	0.24	77	27	0.56
I-III	19	23		33	9	
Stage						
I	42	53	0.57	71	24	0.91
II	10	9		14	5	
III	12	20		25	7	

SqCC, squamous cell carcinoma; AdC, adenocarcinoma.

Table 3. Relationship among TWIST, hypoxia inducible factor (HIF)-1α, carbonic anhydrase (CA) IX, and E-cadherin expression in patients with non-small cell lung carcinomas

Markers		TWIST		p-value
		Negative	Positive	
HIF-1α	Negative	45	43	0.03
	Positive	19	39	
CA IX	Negative	26	30	0.61
	Positive	38	52	
E-cadherin	Negative	39	71	< 0.01
	Positive	25	11	

Survival analysis

The follow-up ranged from 1 to 117 months (mean, 34.7 ± 27.4 months; median, 27.0 months). A univariate analysis was performed by Kaplan-Meier method to evaluate the effect of molecular markers and clinicopathological factors on survival (data not shown). T stage, N stage, and overall stage were significantly correlated with overall survival, whereas HIF-1α, CA IX, TWIST, and E-cadherin expression were not significantly related.

DISCUSSION

The EMT has been recognized as an important process in cancer progression.[1-3] In NSCLC, loss of E-cadherin expression has been recognized as a poor prognostic factor even before the advent of the EMT concept.[26] Now, EMT is an important E-cadherin repressing mechanism. Among EMT inducers, TWIST is a central key player in the EMT of many cancers including NSCLC.[8-12,15-17] Although not fully defined, hypoxia has been implicated as a microenvironmental factor inducing EMT phenomenon.[21-24] In this study, we investigated the expression of endogenous hypoxic markers such as HIF-1α and CA IX, a *CDH1* repressor of TWIST, and E-cadherin in human NSCLC. An inverse relationship between TWIST and E-cadherin has been reported in a variety of cancers including urinary bladder, breast, prostate, and head and neck.[7-12] In the present study, an inverse relationship between TWIST and E-cadherin suggested that TWIST represses E-cadherin expression in NSCLC. Although there are many EMT phenotypic proteins, loss of E-cadherin is a key EMT marker;[1,2] hence, our results indicate that EMT may be related to TWIST expression.

Regarding the relationship between TWIST and hypoxic

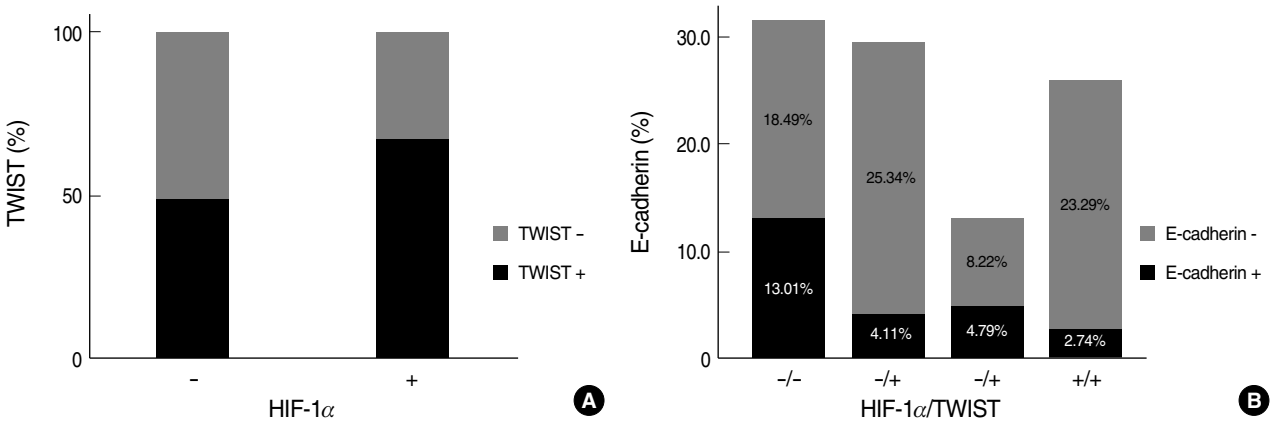

Fig. 3. (A) TWIST expression is higher in the hypoxia inducible factor (HIF)-1α positive group than in the HIF-1α negative group. (B) TWIST expression contributes more to the loss of E-cadherin expression than does HIF-1α expression.

markers, HIF-1α expression correlated with TWIST expression. The odds ratio was 2.1 for the HIF-1α positive group compared to the HIF-1α negative group. However, CA IX expression was not correlated with TWIST expression. We interpreted this finding as follows. Does HIF-1α more precisely reflect a hypoxic environment capable of inducing TWIST expression? However, both HIF-1α and CA IX are well known factors that reflect an endogenous hypoxic condition and are correlated with each other.[20] In this study, HIF-1α and CA IX expression were also correlated, supporting the suggestion that they are endogenous hypoxic markers. The finding that both factors are more frequently expressed in SqCC, which is often exposed to hypoxic conditions, also defines their role as endogenous hypoxic markers.[20,27,28] Hence, we presumed that TWIST is induced transcriptionally through HIF-1α stabilization under hypoxic conditions and is not activated by hypoxia itself, as recent studies have indicated that HIF-1α directly upregulates TWIST expression in head and neck SqCC[22] and directly induces TWIST expression in renal tubular epithelium.[23] Both of these latter studies demonstrated that HIF-1α is stabilized under hypoxic conditions and binds to a heat-responsible element region located in the TWIST gene promoter region. In a study using a pancreatic cancer cell line, TWIST was detected after exposure to hypoxic conditions.[24] Therefore, our results suggest that HIF-1α stabilization by tumor hypoxia contributes to TWIST expression in NSCLC. Although we also expected that a significant relationship between HIF-1α and E-cadherin expression, our results failed to show such an association. When patients were divided into four groups based on HIF-1α and TWIST expression status, the groups showing TWIST positivity were significantly correlated with loss of E-cadherin expression, regardless of HIF-1α status. This finding suggests that TWIST expression mainly contributes to the loss of E-cadherin expression. This unexpected finding could be explained by the observation that many other factors other than hypoxia may be involved in the EMT process. Another possibility is that there was a limitation to detect HIF-1α by immunohistochemistry, because HIF-1α is remarkably unstable and degraded shortly after synthesis.

In the present study, TWIST expression was mainly located in the subcelluar cytoplasmic region and only 14 of 146 cases showed nuclear positivity. We could not determine any significant statistical meaning for the nuclear positivity. In previous studies using immunohistochemistry, TWIST expression was also mainly observed in cytoplasm.[8-12] Cytoplasmic staining was regarded as positive and was inversely correlated with E-cadherin expression. In a study by Yuen *et al.*,[11] nuclear TWIST expression was correlated with metastatic potential in prostatic carcinoma. However, in that study, the nuclear TWIST expression rate was still low (29/152 cases, 19%). Other studies have reported that most embryonic cells express TWIST in their nuclei, whereas differentiated cells express TWIST in the cytoplasm, suggesting that TWIST nuclear-cytoplasmic protein trafficking represses E-cadherin expression at the post-transcriptional regulatory level in cancers.[29,30]

HIF-1α, TWIST, and CA IX expression increased in tumors with a higher tumor grade. We think that tumors with a higher grade tend to be under more hypoxic conditions and that hypoxic marker and TWIST expression increases. In the survival analysis, TWIST expression and loss of E-cadherin expression were not associated with a significant decline in patient survival. The patient groups in this study spanned a 10-year follow-up, although some patients were followed for a shorter time. Studies with more cases and longer follow-up periods will be necessary to evaluate the clinical value of TWIST expression in NSCLC.

In conclusion, the present study demonstrated that HIF-1α expression was associated with TWIST expression. TWIST expression was also inversely correlated with E-cadherin expression. However, a direct relationship between HIF-1α and E-cadherin expression was not observed. This result probably occurred due to many factors, which might be involved in EMT. Although the present study did not show any definite evidence for hypoxia-induced EMT, we suggest that hypoxia may play a potential role to induce TWIST and EMT in NSCLC due to the significant relationship between HIF-1α and TWIST expression. Hence, we suggest that further hypoxia-mediated EMT studies should be conducted.

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