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The Overexpression of Histone Deacetylase 1 and Its Relationship with *p16^{INK4a}* Gene Hypermethylation in Pulmonary Squamous Cell Carcinoma and Adenocarcinoma

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Background : DNA methylation and histone modification are dynamically linked in the epigenetic control of gene silencing and they play an important role in tumorigenesis. **Methods :** To evaluate the role of histone deacetylase 1 (HDAC1) in the development of lung cancer and the relationship between a HDAC1 overexpression and *p16^{INK4a}* hypermethylation, we performed immunohistochemical staining for HDAC1 in 76 lung cancer specimens (39 squamous cell carcinomas and 37 adenocarcinomas) that had been previously evaluated for their *p16^{INK4a}* methylation status by real-time quantitative polymerase chain reaction. **Results :** A HDAC1 overexpression (>50% of HDAC1 immunoreactive cells) was detected in 65 (85.5%) out of the 76 cases and it was more frequently seen in the squamous cell carcinomas (97.4%) than in the adenocarcinomas (73.0%) (p=0.002). The incidence of HDAC1 overexpression tended to be higher in the heavy smokers with more than 20 pack-years (p=0.067). Although there was no statistical significance, the frequency of *p16^{INK4a}* hypermethylation in the cases with a HDAC1 overexpression (27.7%) tended to be higher than that in the cases without a HDAC1 overexpression (9.0%) (p=0.175). **Conclusions :** A HDAC1 overexpression might be involved in lung carcinogenesis, and especially in a subgroup of smoking and squamous cell carcinoma patients, and a HDAC1 overexpression may be associated with *p16^{INK4a}* hypermethylation.

Key Words : Lung; Squamous cell carcinoma; HDAC1 protein, human; Cyclin-dependent kinase inhibitor p16; Hypermethylation

Epigenetic modifications such as DNA methylation, histone modification and RNA-associated silencing are dynamically interconnected and these processes may contribute to the development and progression of cancer.[1] The densely methylated DNA that's associated with transcriptionally repressed chromatin is characterized by the presence of underacetylated histones.[2,3] Thus, methylation and histone deacetylation appear to act as layers for epigenetic gene silencing.

Histone deacetylase (HDAC) deacetylates the acetylated lysine residues within the histone tails, and this causes compaction of the chromatin structure and thus, the repression of gene transcription.[4] In particular, histone deacetylase 1 (HDAC1) is believed to play a key role in the development of various human malignancies.[5-10] An increased HDAC1 expression might lead to hypoacetylation of histones and the subsequent silencing of several tumor suppressor genes. However, there are scarce reports on the presence of the concordant overexpression of HDAC1 at the protein level and its role in lung cancer.

By applying the real-time porlymerase chain reaction (PCR)/SYBR Green detection method, which is a modified method of the previously developed PCR technique,[11] our previous study found a substantial number of samples with an increased methylation status of the promoter region of the *p16^{INK4a}* gene in primary lung cancer specimens.[12] The *p16^{INK4a}* hypermethylation occurred more frequently in the squamous cell carcinoma specimens than in the adenocarcinoma specimens. These findings

spurred us to study the relationship between *p16INK4a* hypermethyaltion and the HDAC1 protein expression in primary lung cancer with a special focus on the histologic type of lung cancer.

We report hereon the correlations between HDAC1 overexpression and the clinicopathological factors and *p16INK4a* hypermethyaltion by using the tissue samples from 76 lung cancer specimens that had been previously studied for their *p16INK4a* methylation status.

MATERIALS AND METHODS

The *p16INK4a* methylation analysis

The DNA extraction from the snap-frozen tumor samples, the bisulfite modification and the *p16INK4a* methylation analysis were performed according to the standard protocols, and as were previously described.[11,12] Briefly, the *p16INK4a* gene methylation status was determined by real-time quantitative PCR followed by restriction enzyme digestion. We used real-time PCR (ABI PRISM 7000 Sequence Detection System, Applied Biosystems, Foster City, USA) to quantify the genomic target sequences with using SYBR Green 2X PCR Master Mix (Applied Biosystems) for detection. One μg of genomic DNA was incubated for 7 days at 37°C with *Msp*I and *Hpa*II (New England BioLab, Beverly, MA, USA). When the external C in the sequence CCGG of the *p16INK4a* gene is methylated, then *Msp*I and *Hpa*II cannot cleave *p16INK4a*. However, unlike *Hpa*II, *Msp*I can cleave this sequence when the internal C residue is methylated. The raw data was evaluated with using ABI 7000 System Software. The methylation status of each sample was expressed as the threshold cycle (C_T) ratio. The C_T ratio reflected methylation and it was calculated as follows: C_T ratio=(C_T of the target gene without treatment-C_T of the target gene treated with *Hpa*II)/(C_T of the target gene without treatment-C_T of the target gene treated with *Msp*I). In a totally non-methylated state, the C_T ratio of the *p16INK4a* gene is 1, and so a lower C_T ratio reflects a higher level of methylation. The human cell line Wi-38 (KCLB no. 10075.1, epithelium of lung) was used as a positive control for the methylated alleles. Water blanks were used as a negative control.

Patient selection for HDAC1 immunohistochemistry

During this study, we analyzed 76 cases (39 squamous cell carcinomas and 37 adenocarcinomas) of the 81 primary lung cancers that we had previously studied for determining their *p16INK4a* methylation status. The tissues were obtained from patients who were operated on at Dong-A University Medical Center from 2006 to 2007. No preoperative chemotherapy or radiotherapy had been performed in any of these cases. Standard lobectomy and lymph node dissections were performed in every case. The institutional review board approved our study, and written informed consent was obtained from all the patients for surgery and to use their resected samples for research. The hematoxylin and eosin-stained slides were reviewed in each case to confirm the original diagnosis, and this was based on the World Health Organization criteria.[13] The clinical records, the pathological reports and the clinical follow-up information were also obtained, when available. The postoperative pathological staging was determined according to the guidelines of the American Joint Committee on Cancer (AJCC).[14]

Immunohistochemistry for HDAC1

Immunohistochemical study for HDAC1 was performed on the formalin-fixed, paraffin-embedded, 4 μm-thick tissue sections with using the avidin-biotin-peroxidase complex method. Mouse monoclonal antibody was used at a 1:200 dilution as the primary antibody directed against HDAC1 (LabVision, Fremont, CA, USA). Deparaffinization of all the sections was performed through a series of xylene baths, and rehydration was performed with a series of graded alcohol solutions. To enhance the immunoreactivity, microwave antigen retrieval was performed at 750 W for 30 min in Tris-EDTA buffer (pH=9.0). After blocking the endogenous peroxidase activity with 5% hydrogen peroxidase for 10 min, primary antibody incubation was performed for 1 h at room temperature. The antibody in an Envision™ Chem™ Detection Kit (DakoCytomation, Carpinteria, CA, USA) was used for the secondary antibody at room temperature for 30 min. After washing the tissue samples in Tris buffered saline for 10 min, 3, 3′-diaminobenzidine was used as a chromogen and then Mayer's hematoxylin counterstain was applied.

Interpretation of the immunohistochemical staining

A semiquantitative assessment of HDAC1 staining on the whole-tissue sections was performed independently by two observers (K.E.L. and M.S.R.), who were kept "blinded" to all the clinicopathologic variables. The score of each whole tissue section was based on the frequency, regardless of the intensity, of the cells with positive nuclear staining (range: 0% to 100%) within the tumor. If immunoreactivity was present, then the expression of

HDAC1 was usually diffuse and strong. Therefore, HDAC1 was considered to be overexpressed when the distribution of the stained cells amounted to more than 50% of the total tumor cells.

Statistical analysis

The data was analyzed with SPSS 14.0 software (Chicago, IL, USA). The associations between HDAC1 overexpression and the clinicopathological characteristics and the $p16^{INK4a}$ methylation status were analyzed by using contingency tables. Statistical significance was evaluated with chi-squared tests. Statistical differences were considered significant for p-values less than 0.05.

RESULTS

The clinicopathological characteristics

The patients consisted of 60 (78.9%) men and 16 (21.1%) women, and they ranged in age from 31 to 75 years (median age: 63 years). Fifty-six patients (73.7%) had a smoking history. The tumor sizes ranged from 1 cm to 11 cm (median size: 3.7 cm); 34 (44.7%) cases had tumors ≤3 cm in size and 42 (55.3%) cases had tumors >3 cm in size. Histologically, the tumors consisted of 39 (51.3%) squamous cell carcinomas and 37 (48.7%) adenocarcinomas. Lymph node metastases were present in 35 (46.1%) of the 76 cases, and lymphovascular tumor invasion was seen in 39 (51.4%) of the 76 cases. Seventeen (22.3%) cases were at T1,

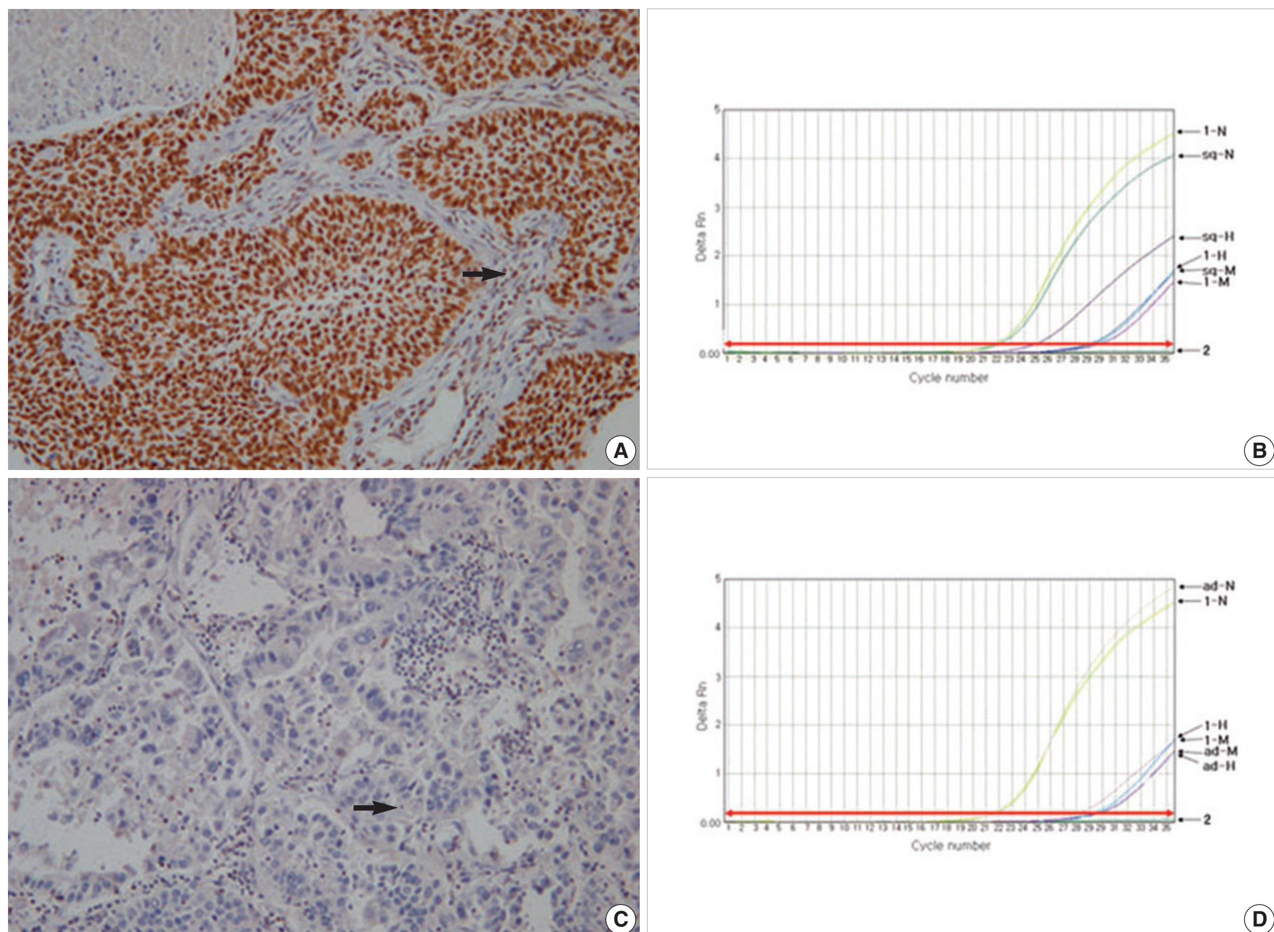

Fig. 1. Immunohistochemical study for histone deacetylase 1 (A, C) and $p16^{INK4a}$ methylation study by performing real-time polymerase chain reaction/SYBR Green detection method (B, D) in lung cancer specimens. A representative case of squamous cell carcinoma shows overexpressed histone deacetylase 1 with a nuclear reaction (A) and $p16^{INK4a}$ hypermethylation (B), whereas a representative case of adenocarcinoma shows a negative reaction for histone deacetylase 1 (C) and an unmethylated $p16^{INK4a}$ gene (D). Arrows, interstitial lymphocytes showing positive expression for HDAC1 as internal positive control; sq, methylated squamous cell carcinoma; ad, unmethylated adenocarcinoma; 1, positive control (wi-38); 2, negative control (water); N, no-cut DNA amplification; H, *Hpa* II-cut DNA amplification; M, *Msp* I-cut DNA amplification; Delta Rn, the magnitude of the fluorescence signal generated during the PCR at each time point.

44 (57.9%) were at T2, 11 (14.5%) were at T3 and 4 (5.3%) were at T4. Thirty-seven (48.7%) cases were at stage I, 10 (13.2%) were at stage II, 27 (35.5%) were at stage III and 2 (2.6%) were at stage IV.

The *p16*^{INK4a} methylation status and its relationship with the clinicopathological factors

The detailed results were previously described elsewhere.[6] Briefly, the frequency of *p16*^{INK4a} hypermethylation was 25.0% (19 cases) for the 76 cases of lung cancer. The incidence of *p16*^{INK4a} hypermethylation was significantly higher in the squamous cell carcinomas (13/39, 33.3%) than that in the adenocarcinomas (6/37, 16.2%) (p=0.048). Importantly, the *p16*^{INK4a} hypermethylation had a positive association with the number of pack-years smoked (methylated: 37.4±22.3 pack-years vs. unmethylated: 25.2±22.0 pack-years) (p=0.043) and the duration of smoking (p=0.048). There were no significant associations between the *p16*^{INK4a} methylation status and the age and gender of the patients and the AJCC stage.

Table 1. The relationship between the clinicopathological characteristics and the histone deacetylase 1 overexpression in 76 lung cancers

Clinicopathological characteristics	No. of cases	HDAC1 overexpression n=65 (%)	p
Age			0.747
≤60	33	29 (87.9)	
>60	43	36 (83.7)	
Gender			0.882
Male	60	51 (85.0)	
Female	16	14 (87.5)	
Histologic type			0.002
Squamous cell carcinoma	39	38 (97.4)	
Adenocarcinoma	37	27 (73.0)	
Smoking status			0.465
Never	20	16 (80.0)	
Ever	56	49 (87.5)	
Tobacco consumption			0.067
0	20	16 (80.0)	
≤20 pack-years	8	5 (62.5)	
>20 pack-years	48	44 (91.7)	
Tumor size			0.526
≤3 cm	34	28 (82.4)	
> 3 cm	42	37 (88.1)	
Lymph node metastasis			0.543
Negative	41	36 (87.8)	
Positive	35	29 (82.9)	
Lymphovascular invasion			0.817
Negative	37	32 (86.5)	
Positive	39	33 (84.6)	
T stage			0.148
1	17	14 (82.4)	
2	44	40 (90.9)	
3	11	9 (81.8)	
4	4	2 (50.0)	
AJCC stage			0.398
I	37	33 (89.2)	
II	10	9 (90.0)	
III	27	22 (81.5)	
IV	2	1 (50.0)	

HDAC1, histone deacetylase 1; AJCC, American Joint Committee on Cancer.

The immunohistochemical findings of HDAC1 and its relationship with the clinicopathologic factors

The expression of HDAC1 protein was detected in the nuclei of both the normal cells and the tumor cells. In the adjacent normal lung, the nonneoplastic bronchial and alveolar epithelial cells, the endothelial cells and the interstitial lymphocytes were heterogeneously reactive for HDAC1. However, the surface ciliated and mucous cells of the bronchial epithelium and the seromucinous glands of the bronchial wall did not show HDAC1 immunostaining. In the tumor tissue, HDAC1 overexpression was detected in 65 (85.5%) out of the 76 cases. Thirty-eight (97.4%) of the 39 squamous cell carcinomas and 27 (73.0%) of the 37 adenocarcinomas showed HDAC1 overexpression. The HDAC1 overexpression was significantly associated with the histologic type (p=0.002) (Fig. 1). Although the difference was not statistically significant, the incidence of a HDAC1 overexpression was higher in the heavy smokers with more than 20 pack-years smoked (p=0.067). However, there were no significant associations between the HDAC1 overexpression and the age and gender of the patients, the tumor size, the presence of lymph node metastasis, lymphovascular invasion, the T stage and the AJCC stage (Table 1).

The relationship between the HDAC1 overexpression and the *p16*^{INK4a} methylation status

Eighteen (27.7%) of the 65 cases and 1 (9.1%) of the remain-

Table 2. Relationship between the histone deacetylase 1 overexpression and *p16*^{INK4a} methylation status in 76 lung cancers

p16^{INK4a}	HDAC1 overexpression		p
	Absent n=11 (%)	Present n=65 (%)	
Unmethylated (n=57)	10 (91.0)	47 (72.3)	0.175
Methylated (n=19)	1 (9.0)	18 (27.7)	

HDAC1, histone deacetylase 1.

ing 11 cases with and without a HDAC1 overexpression showed $p16^{INK4a}$ hypermethylation. Although there was no statistical significance, the frequency of $p16^{INK4a}$ hypermethylation in the cases with a HDAC1 overexpression tended to be higher than that in those cases without a HDAC1 overexpression for all 76 of the studied cases (p=0.175) (Fig. 1) (Table 2).

DISCUSSION

The findings of this study showed that the HDAC1 overexpression was more significantly increased in squamous cell carcinoma than in adenocarcinoma of the lung. Furthermore, the incidence of HDAC1 overexpression tended to be higher in the heavy smokers with more than 20 pack-years smoked. Our previous study on the $p16^{INK4a}$ methylation status also showed that $p16^{INK4a}$ hypermethylation occurred more frequently in squamous cell carcinoma than in adenocarcinoma, and this had a positive association with the number of pack-years smoked and the duration of smoking,[12] which is consistent with the findings of other studies.[15-18] Pulmonary squamous cell carcinomas are closely related to cigarette smoking and most of these arise from the larger airway, whereas pulmonary adenocarcinomas are less closely related to cigarette smoking and most of these arise from the peripheral lung.[19] Epigenetic modifications seem to have different importance in the development of different types of lung tumor. Lee *et al.*[16] demonstrated that nickel, a carcinogenic metal found in cigarette smoking, silenced the gpt expression in Chinese hamster ovary cells via DNA methylation, and nickel induced the chromatin to condense and it caused heterochromatization of the gpt integration site. These results imply that the association between HDAC1 overexpression, $p16^{INK4a}$ methylation and smoking may be clinically relevant and these events create a permissive environment for lung cancer to develop, and especially for the development of squamous cell carcinoma.

In regard to the role of HDAC1 overexpression in the biologic behavior of lung cancer, in this study there were no significant associations between the HDAC1 overexpression and the pathologic factors that showed tumor progression, including lymph node metastasis, lymphovascular invasion and the tumor stage. Our results are in general agreement with those of a previous study that focused on the HDAC1 expression levels in colorectal carcinomas.[6] However, Sasaki *et al.*[8] showed that the HDAC1 mRNA expression levels were significantly increased in the advanced stage of lung cancers. Our study is limited by the number of study cases and by the relatively small subgroups. Further investigations with a larger number of cases and a longer follow up period would allow us to evaluate HDAC1 in a variety of clinical settings, and this would help us better understand the impact of HDAC1 on the biological behavior of lung cancer.

DNA-methylation-induced chromatin remodeling that's facilitated by HDACs activity is now considered to be one of the important factors in the repression of various genes during malignant transformation.[1] However, the mechanisms underlying these observations are still obscure. The evidence that several methyl CpG-binding proteins (e.g., MeCP2 and MBD2) interact with HDAC and recruit corepressor proteins lends support to a mechanism that links DNA methylation and histone modification.[20,21] Although there was no statistical significance, the frequency of $p16^{INK4a}$ hypermethylation in the cases with a HDAC1 overexpression tended to be higher than that in those cases without a HDAC1 overexpression for all 76 of the studied cases. The altered methylation pattern of $p16^{INK4a}$ and the HDAC1 overexpression observed in this study may have implications for understanding the roles of epigenetic interaction in the pathogenesis of lung cancer.

The epigenetic changes involved in developing cancer are reversible, which is unlike the genetic changes; therefore, this has recently become a target for novel anticancer drugs. HDAC inhibitors can induce differentiation, growth arrest and/or apoptosis in transformed cells in vitro and in tumors.[1] Cantor *et al.*[22] showed that treatment of FHIT-deficient human non-small cell lung cancer H1299D1 cells with hypomethylating monotherapy or with combined hypomethylating and histone deacetylating therapy rendered the cells nontumorigenic, although the drugs did not effectively reach many of the cells in the large solid tumor of the lung cancer xenograft model. They suggested that demethylating and histone acetylation, singly or in combination, could be useful for treating lung cancer patients with local spread to the lymph nodes and for whom their large tumors had been removed by surgery. Our results may lay a helpful foundation for the use of novel hypomethylating agents and/or HDAC inhibitors to treat a subgroup of smoking and squamous cell carcinoma patients, who reveal frequent HDAC1 overexpression and $p16^{INK4a}$ hypermethylation.

We conclude that a HDAC1 overexpression might be involved in lung carcinogenesis, and especially in a subgroup of smoking and squamous cell carcinoma patients, and this may be associated with $p16^{INK4a}$ hypermethylation. Further studies will be needed to clarify the downstream mechanisms that are involved in controlling the biological behavior of lung cancer via histone-modifying molecules. In addition, such information may help

us create novel therapeutic strategies for treating primary lung cancer.

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