

Differential Expression of Hypoxia Inducible Factor-1 α and Tumor Cell Proliferation Between Squamous Cell Carcinomas and Adenocarcinomas Among Operable Non-Small Cell Lung Carcinomas

This study aimed to evaluate whether the elevated level of hypoxia-inducible factor-1 α (HIF-1 α) correlated with histologic types, angiogenesis, tumor cell proliferation, and clinical parameters in common non-small cell lung carcinomas (NSCLCs). We performed immunohistochemical stains using paraffin-embedded tissue blocks from 84 cases of operable NSCLC [No. of squamous cell carcinoma (SCC), 45; No. of adenocarcinoma (AC), 39]. HIF-1 α expression was related with histologic types (66.7% in SCCs vs 20.5% in ACs, $p < 0.001$), but not with lymph node status, tumor stage, vascular endothelial growth factor expression, microvessel density (MVD), and proliferating cell nuclear antigen (PCNA) index ($p > 0.05$, respectively). As for the histologic types, MVD and PCNA index were significantly higher in SCCs than in ACs ($p = 0.009$ and $p = 0.016$, respectively). Among HIF-1 α positive carcinomas, MVD was significantly higher in HIF-1 α positive SCCs than in HIF-1 α positive ACs ($p = 0.023$). The overall survival curves were not associated with HIF-1 α expression or any other histologic parameters ($p > 0.05$). These findings suggest that HIF-1 α expression in NSCLCs may play a differential role according to histologic types, but its prognostic significance is indeterminate.

Key Words : Carcinoma, Non-Small Cell Lung; Hypoxia-inducible Factor-1 α ; Endothelial Growth Factors; Antigens, CD34; Proliferating Cell Nuclear Antigen; Immunohistochemistry

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INTRODUCTION

Cells, whether normal or malignant, have the ability to 'sense' low oxygen condition, probably via a heme flavo-oxidoreductase protein or even through hypoxia-stimulated release of reactive oxygen species from mitochondria (1), which activates a signaling pathway for the expression of the hypoxia-regulated genes. A widespread oxygen sensing system exists in mammalian cells. This regulates the expression of a diverse group of genes including erythropoietin, glucose transporters, glycolytic pathway enzymes, vascular endothelial growth factor (VEGF), transferrin, heme oxygenase, and inducible nitric oxide synthetase, many of which are known to be up-regulated in cancer (2). In tumor cell lines, hypoxia-regulated gene expression has been shown to involve the stabilization, nuclear accumulation, and DNA binding of the transcription factors hypoxia inducible factors (HIFs). These proteins are heterodimers consisting of two different, hypoxia-inducible alpha subunits (HIF-1 α and HIF-2 α) and a common, constitutive beta subunits (HIF-1 β) identical to aryl hydrocarbon nuclear receptor translocator (ARNT) (3, 4). The critical determinant of HIF activity is the level of HIF-1 α or HIF-2 α protein, since ARNT or HIF-1 β is constitutively present (5). On expo-

sure to hypoxia, both HIF-1 α and HIF-2 α proteins accumulate rapidly in the nucleus and bind to short DNA sequences called hypoxia-response elements near or in oxygen-sensitive genes, stimulating gene expression. Although the exact mechanism of oxygen sensing remains to be elucidated, the cell probably senses its oxygen concentration through reactive oxygen species, so that stabilization of HIF-1 α or HIF-2 α protein is said to be redox induced (6). With reoxygenation, they disappear and their cellular levels are determined mainly by the rate of ubiquitin-proteasomal degradation (7).

Regions of hypoxia are known to exist within many tumors, and the extent of tumor hypoxia correlates with prognosis in a number of tumor types (8, 9). Hypoxia in the tumor microenvironment is sufficient to activate HIF-dependent gene expression (10). A major role of HIF proteins in determining gene expression, tumor angiogenesis, and growth has been demonstrated in xenograft experiments with a cell line deficient in ARNT (11). Description of the distribution within human tissue of HIF-1 α has primarily been of mRNA. But the nuclear accumulation of HIF-1 α protein can be also detected immunohistochemically and has been shown to occur in human malignancies and their metastases (12). Recently Giacomolaki et al. (13) reported that the immunohistochemi-

cal expression of HIF-1 α and HIF-2 α proteins in non-small cell lung carcinomas (NSCLCs) was related to the up-regulation of various angiogenic factors including VEGF and with poor prognosis. They described that HIF-1 α and HIF-2 α expression was observed in both cytoplasmic and nuclear portions, and regarded both expression patterns as positivity for HIF-1 α and HIF-2 α proteins (13). However, the impact of HIF-1 α protein on tumor growth kinetics of NSCLC is not still defined.

In the present study we studied the differential immunohistochemical expression of HIF-1 α protein according to common histologic types of NSCLCs when only nuclear HIF-1 α staining was adopted as positivity. In addition, HIF-1 α expression was also analysed in comparison with microvessel density (MVD), VEGF expression, proliferating cell nuclear antigen (PCNA) index, and clinical prognostic parameters in NSCLCs.

MATERIALS AND METHODS

Eighty-four NSCLC samples used in this study were obtained from patients who underwent lobectomy or pneumonectomy for operable lung cancers. They all were diagnosed at the Department of Pathology, Pusan National University Hospital between 1998 and 2000. The tumors were composed of 45 cases of squamous cell carcinoma (SCC) and 39 of adenocarcinoma (AC).

The latter was composed of acinar or papillary ACs of peripheral type. Seven cases of them showed focal bronchioloalveolar carcinoma (BAC) pattern. As for ACs with BAC pattern, separate subtyping was not performed because they were generally known to show similar biologic behavior with usual ACs.

Resected lung tissues were fixed immediately in 10% buffered formalin (pH 7.0) and embedded in paraffin, and 4 μ m-thick serial sections were prepared. For histopathological diagnosis, one of these sections was stained with hematoxylin-eosin. The others were used for immunohistochemistry. The pathological diagnosis was based on light microscopic examination, according to the WHO classification (14).

Immunohistochemical staining

Sections from paraffin-embedded blocks were transferred to poly-L-lysine coated glass slides and air-dried overnight at 37°C. They were dewaxed in xylene (three changes), rehydrated in a graded series of decreasing ethanol concentrations, and then rinsed in Tris-buffered saline (50 mM Tris/HCl, pH 7.4, containing 100 mM sodium chloride). For the blockage of endogenous peroxidase activity, sections were immersed for 20 min in methanol containing 0.3% hydrogen peroxide. Table 1 presents the summary of immunohistochemical panel used in this study. For the detection of HIF-1 α , tissue sections were immersed in 10 mmol/L citrate buffer (pH 6.0), and subjected to microwave irradiation for 5 min \times 3. After antigen

retrieval, a cooling-off period of 30 min was followed, and HIF-1 α antibody was incubated with the tissue sections one hr at 20°C in moisture chamber. Thereafter, the catalysed signal amplification system (DAKO Co., Carpinteria, CA, U.S.A.) was used according to the manufacturer's instructions. All other antibodies to CD34, VEGF and PCNA were used without antigen retrieval treatment. They were incubated with the tissue sections overnight at 4°C, and then immunohistochemical procedures were performed by Histostain Plus kit (Zymed Laboratories Inc., South San Francisco, CA, U.S.A.) using the standard streptavidin-biotin complex method. The reaction products were visualized by exposing sections to 3,3'-diaminobenzidine for HIF-1 α , and to 3-amino-9-ethylcarbazole for the others. Nuclei were lightly counterstained for about 20 sec with Meyer's hematoxylin. Sections were then mounted in diluted malinol after the application of Universal Mount (DAKO, Carpinteria, CA, U.S.A.). Tissue samples incubated with non-immune serum served as negative controls. Breast cancer tissue sections with strong nuclear HIF-1 α expression were used as positive controls.

Immunohistochemical interpretation

All immunohistochemical evaluation was performed by two independent observers. Interobserver variability was minimal ($p < 0.05$ by χ^2 test). HIF-1 α staining was regarded as positivity if the tumor cells of more than 1% within tumor tissue showed completely darkly stained nuclei. Cytoplasmic staining, observed occasionally, was ignored because active HIF-1 α is located only in the nucleus (15). Angiogenic activity was assessed by estimating CD34-positive microvessels in the surrounding stroma of invasive tumor nests as described in the literature (16). In short, in four adjacent fields of vision in the most vascularized area, microvessels were counted at $\times 200$ magnification using an Olympus microscope (BX 50, Olympus Optical Co., Japan), and then MVD was expressed as the mean value of microvessels/mm² for each case. For PCNA evaluation, we examined at least 500 tumor cells, to determine whether the nuclei of the cells were positive for the PCNA staining at high power ($\times 400$) after screening for areas of highest intense staining at low power ($\times 100$). We did not divide the

Table 1. Immunohistochemical panel used in this study

Antigen	Antibody clone	Dilution	Antigen retrieval	Source
HIF-1 α	Mouse Mab (H1a67)	1:1,000	MW	Novus*
CD34	Mouse Mab (QBEnd/10)	1: 50	ND	NeoMarkers [†]
VEGF	Goat poly (A20)	1: 50	ND	Santa Cruz [‡]
PCNA	Mouse Mab (PC10)	1: 1,500	ND	Sigma [§]

MAB, monoclonal antibody; poly, polyclonal antibody; MW, microwaving with 10 mM/L citrate buffer, pH 6.0 (3 \times 5 min.); ND, not done; *, Novus Biological Inc., Littleton, CO, USA; [†], NeoMarkers, Union City, CA, USA; [‡], Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; [§], Sigma Chemical Co., St. Louis, Mo, USA.

PCNA-expression level. Instead we used the percentage of PCNA-positive cells (PCNA index) for all analyses. For evaluation of VEGF expression a score corresponding to the sum of both (a) staining intensity (0, negative; 1, weak; 2, intermediate; 3, strong) and (b) percentage of positive cells (0, 0% positive cells; 1, <25% positive cells; 2, 26-50% positive cells; 3, >50% positive cells) was determined. Their sum reached a maximum score of 6. A score greater than 2 was regarded as positive (17).

Statistical analysis

To evaluate whether the elevated level of HIF-1 α correlated with histologic types, VEGF expression, lymph node status, and clinical stages, we performed χ^2 test for trend. Associations between HIF-1 α expression and PCNA index and MVD were analysed with Student t-test. Within the cases of HIF-1 α positive NSCLC, we also evaluated the relationship between histologic types and VEGF expression by χ^2 test and between histologic types and PCNA index and MVD by Mann Whitney U test. Finally with relation to histologic parameters including HIF-1 α expression, histologic type, VEGF expression, MVD, and PCNA index, each survival curve was analyzed using the Kaplan-Meier methodology. Then the log-rank test was used to determine statistical differences between life tables. All analyses were performed with the SPSS statistical package on a personal computer, Release 10.0.1 (SPSS Inc., Chicago, IL). *p* values less than 0.05 were regarded as statistically significant. All statistical tests were two-sided.

RESULTS

Relationship between HIF-1 α expression and clinicopathologic parameters in SCCs and ACs of lung

Immunoreactivity of HIF-1 α was found in 38 of 84 cases of pulmonary SCCs and ACs (45.2% in positivity). For histologic type, SCCs showed HIF-1 α positivity in 30 out of 45 cases (66.7%), whereas ACs showed the positivity in 8 out of 39 cases (20.5%). HIF-1 α expression was significantly different between the two histologic types ($p < 0.001$; Table 2). Among

Table 2. Relationship between HIF-1 α expression and clinicopathologic parameters in squamous cell carcinomas and adenocarcinomas of the lung

Parameters	Total (No. of cases)	HIF-1 α		<i>p</i> value	
		Negative	Positive		
Histologic types	SCC	45	15	30	<0.001
	AC	39	31	8	
LNM	Yes	34	20	14	0.753
	No	47	26	21	
Stage	I	39	20	19	0.570
	II	14	8	6	
	III	28	18	10	
VEGF	Negative	27	18	9	0.131
	Positive	57	28	29	
MVD (mm ²)*	84	17.8 \pm 10.1	20.5 \pm 9.7	0.215	
PCNA (%) [†]	84	46.2 \pm 22.0	54.9 \pm 22.3	0.077	

SCC, squamous cell carcinoma; AC, adenocarcinoma; LNM, lymph node metastasis; MVD, microvessel density. * and [†], expressed as mean \pm SD.

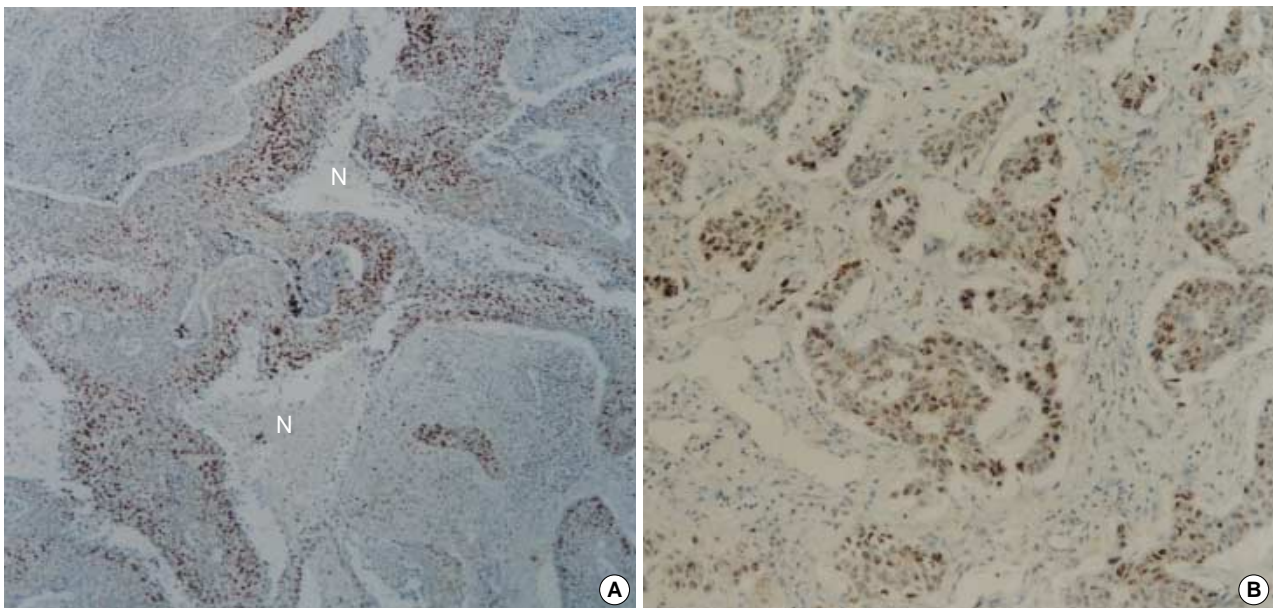


Fig. 1. Immunostaining for HIF-1 α protein. (A) Squamous cell carcinoma shows dark brown nuclear staining, predominantly around necrotic area of the tumor ($\times 40$). (B) Moderately differentiated adenocarcinoma reveals positive nuclear reaction in a randomly scattered fashion ($\times 100$). N, necrotic area.

39 SCC cases showing HIF-1 α positivity, 22 cases exhibited the positive reaction predominantly around necrotic zones of tumor nests (Fig. 1A), and the others revealed the positivity in both around necrosis and non-necrotic area. In comparison with SCCs, five of the positive eight cases of ACs revealed the positive reaction only in non-necrotic areas (Fig. 1B), and three of them showed the combined positive reaction in both non-necrotic areas and around necrotic zones of tumor nests (Table 3). In ACs the positive reaction displayed the tenden-

Table 3. Distribution of HIF-1 α staining in squamous cell carcinomas and adenocarcinomas of the lung

Histologic types	Distribution of HIF-1 α staining		
	Only around necrosis	Only non-necrotic area	Combined*
SCC	22	0	8
AC	0	5	3

SCC, squamous cell carcinoma; AC, adenocarcinoma. *, positive nuclear staining presenting in both around necrosis and non-necrotic area.

Table 4. VEGF expression, MVD, and PCNA index in squamous cell carcinomas and adenocarcinomas of the lung

Histologic types	VEGF (No. of cases)		MVD (/mm ²)	PCNA (%)
	Negative	Positive		
SCC	14	31	21.7 \pm 9.4	55.6 \pm 21.9
AC	13	26	16.0 \pm 9.8	43.9 \pm 21.6
<i>p</i> value	0.828		0.009	0.016

SCC, squamous cell carcinoma; AC, adenocarcinoma; MVD, microvessel density.

cy to occur in histologically poorly differentiated tumor portions. In both histologic types, HIF-1 α positive tumor cells was focally scattered in small clusters. In contrast to SCCs, ACs more frequently showed cytoplasmic immunoreactivity which was regarded as negative reaction. Some non-neoplastic respiratory epithelial tissues neighboring to carcinomas also displayed focal nuclear positive reaction.

With the whole cases of this study, HIF-1 α expression was not correlated with lymph node status, tumor stage, VEGF expression, MVD, or PCNA index ($p > 0.05$, respectively; Table 2).

VEGF expression, MVD, and PCNA index according to SCCs and ACs of lung

Immunoreactivity for VEGF showed no association between the two histologic types (68.9% in SCCs vs 66.7% in ACs, $p = 0.828$; Table 4). In the positive cases the reaction was noted in the cytoplasm of tumor cells over the large areas of tumors (Fig. 2). In addition to tumor cells, stromal cells also displayed positive reaction. CD34 immunoreactivity for MVD was heterogeneous, with no difference between the central

Table 5. VEGF expression, microvessel density, and PCNA index according to HIF-1 α positive squamous cell carcinomas and adenocarcinomas of the lung

Histologic types	VEGF (Positivity, %)	MVD (/mm ²)	PCNA (%)
SCC	24/30 (80.0)	21.5 \pm 8.5	54.2 \pm 20.7
AC	5/8 (62.5)	12.7 \pm 7.4	42.2 \pm 24.4
<i>p</i> value	0.363	0.023	0.227

SCC, squamous cell carcinoma; AC, adenocarcinoma; MVD, microvessel density.

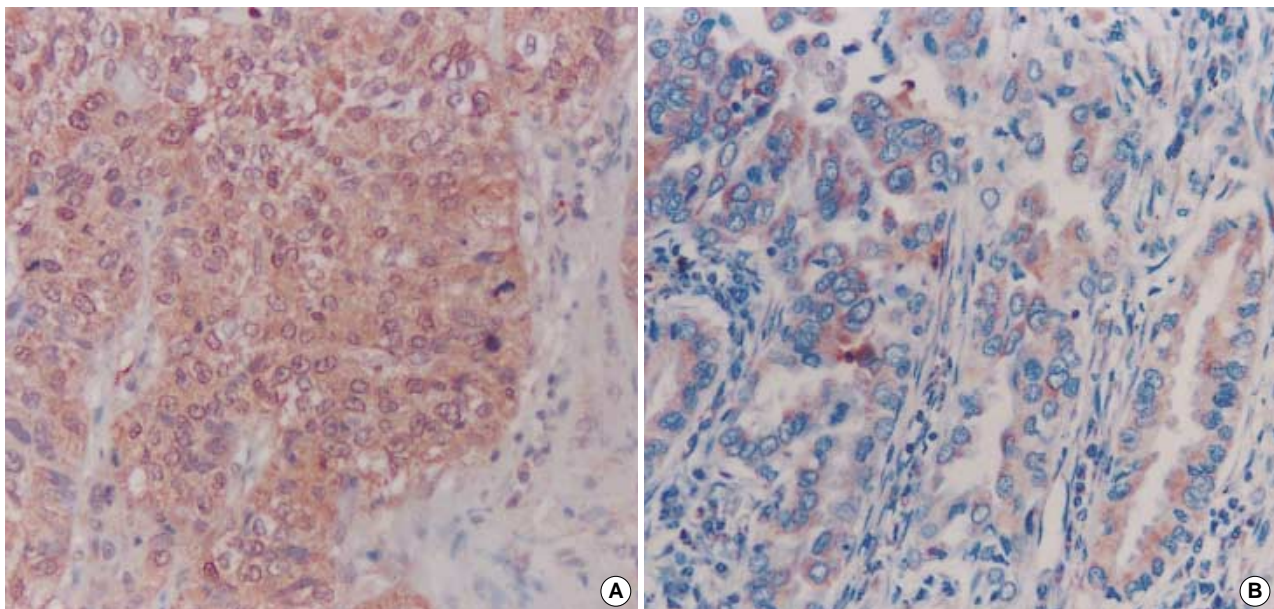


Fig. 2. Immunostaining for VEGF. (A) Squamous cell carcinoma shows reddish brown cytoplasmic staining in a diffuse fashion ($\times 200$). (B) Adenocarcinoma reveals the positive cytoplasmic reaction in a rather focal pattern ($\times 200$).

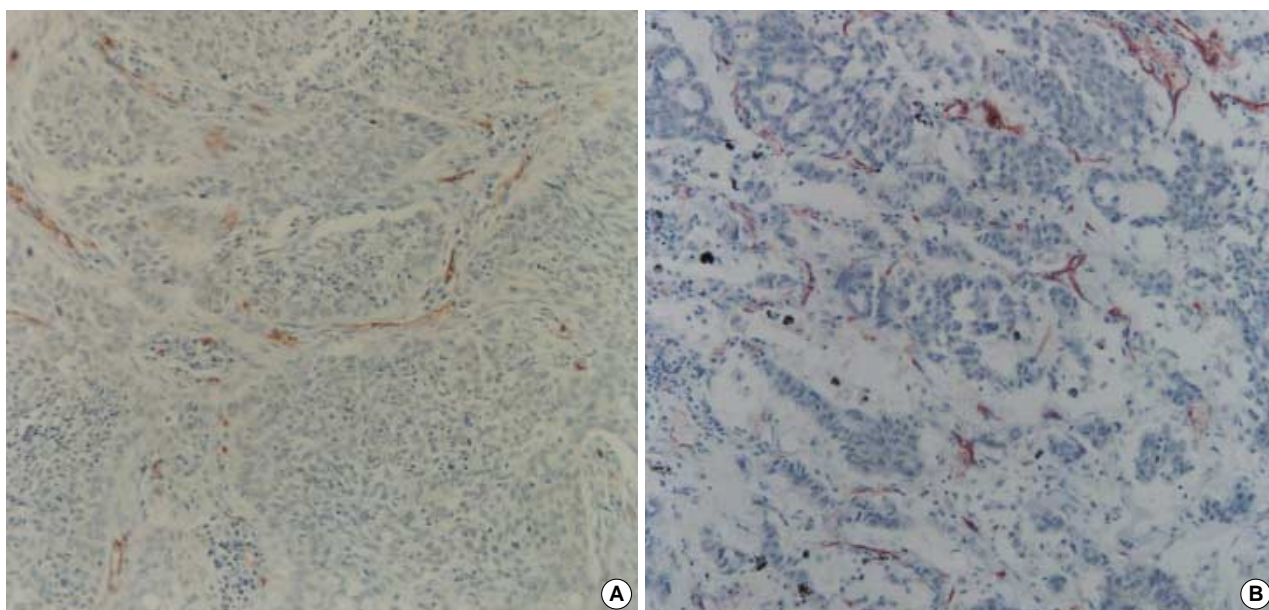


Fig. 3. Immunostaining for CD34. (A) Squamous cell carcinoma shows increased CD34-positive microvessels in the interfaces between carcinoma nests and stoma ($\times 100$). (B) Adenocarcinoma also reveals increased CD34-positive microvessels around the tumor ($\times 100$).

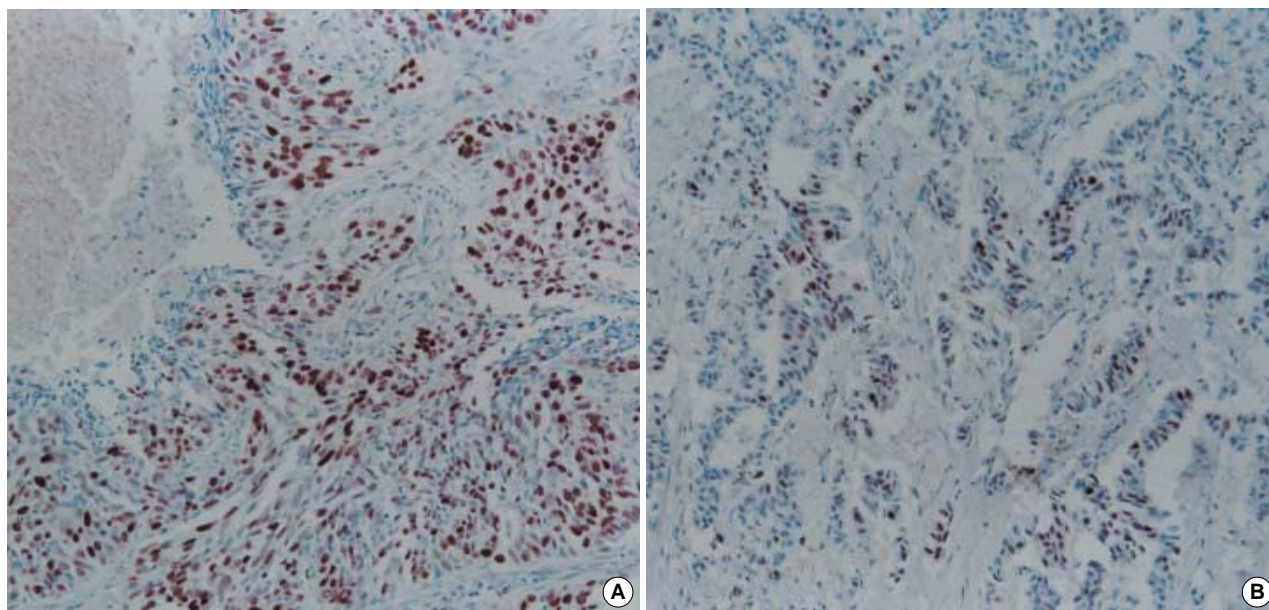


Fig. 4. Immunostaining for PCNA. (A) Squamous cell carcinoma shows diffuse strong positivity in the nuclei of the tumor cells ($\times 100$). (B) Adenocarcinoma reveals the positive nuclear reaction in a rather focal pattern ($\times 100$).

and marginal tumor areas. MVD revealed a significant difference between the two histologic types (21.7 ± 9.4 microvessels/ mm^2 in SCCs vs 16.0 ± 9.8 microvessels/ mm^2 in ACs, $p=0.009$; Fig. 3; Table 4). The PCNA index also showed a significant difference between the two histologic types ($55.6 \pm 21.9\%$ in SCCs vs $43.9 \pm 21.6\%$ in ACs, $p=0.016$; Fig. 4; Table 4).

VEGF expression, MVD, and PCNA index according to HIF-1 α positive SCCs and ACs

VEGF expression was present in 24 of 30 cases (80.0%) of HIF-1 α positive SCCs, and in 5 of 8 cases (62.5%) of HIF-1 α positive ACs. Compared with HIF-1 α positive AC, HIF-1 α positive SCCs showed the tendency of higher VEGF expression, but there was no association between histologic types

($p=0.363$; Table 5). MVD was significantly higher in HIF-1 α positive SCCs than in HIF-1 α positive ACs ($p=0.023$; Table 5). The PCNA index turned out to exhibit no difference between the two histologic types showing HIF-1 α positivity ($p=0.227$; Table 5).

Survival analysis

During the course of this study (maximal follow-up, 132 months; minimal follow-up, 1 month; median follow-up, 23 months), 9 cases were lost to follow-up. In this analysis all patients died of the disease, and data on postoperative treatment were not available. As for MVD and PCNA index, we used the median values (17.8 microvessels/mm² and 43.0%, respectively) as cut-off points in the univariate analysis of survival.

Firstly, with regard to HIF-1 α expression, 14 of the positive 34 patients were censored and 20 died (mean and median survival times, 47.3 and 29.0 months, respectively). Nine of the negative 41 patients were censored and 32 died (mean and median survival times, 36.9 and 24.0 months, respectively). The overall survival was not associated with HIF-1 α expression ($p=0.442$ by log-rank test). It also had no relation with histologic types, VEGF expression, MVD, or PCNA index ($p=0.672$, $p=0.277$, $p=0.890$, and $p=0.573$ by log-rank test, respectively).

DISCUSSION

HIF-1 α protein is known to activate the transcription of genes encoding transferrin, VEGF, endothelin-1, and inducible nitric oxide synthetase, which are implicated in vasodilation, neovascularization, and tumor metastasis, and plays an essential role in oxygen homeostasis (2, 18, 19). In the present study, we investigated the differential expression of HIF-1 α protein among common histologic types of NSCLC. In the cases of SCC, HIF-1 α positive cells were predominantly located around tumor necrosis. The predominant perinecrotic expression of HIF-1 α protein indicates that the hypoxic tumor microenvironment may directly contribute for induction of HIF-1 α activity in these cancers. Nevertheless, some positively staining cells were also present in non-necrotic tumor nests. In the lower parts of columnar or squamous metaplastic epithelium neighboring to the tumor, the focal expression of HIF-1 α protein was also observed, which would be consistent with the presence of low pH and hypoxia. In the cases of AC, however, it was interesting that the increased level of HIF-1 α protein was noted more frequently in poorly differentiated areas than in necrotic areas. This finding may reflect the existence of alternative regulatory modes of HIF-1 α expression. Altered patterns of gene expression in cancer could arise both from genetic alterations in the tumor cells and from stimulation by an abnormal microenvironment within the tumor. As a matter of fact, a growing line of evidence indicates that both

oncogene activation and tumor suppressor gene inactivation are also associated with increased HIF-1 α expression (20-22). In other words the persistent HIF-1 α expression in poorly differentiated ACs may show the emergence of an aggressive phenotype with high oxygen consumption as a result of the transformation itself and not of the hypoxic environment. HIF-1 α expression has been shown to be enhanced by v-src (20) and in response to several growth factors, including insulin-like growth factors (IGFs) 1 and 2, basic fibroblast growth factor, and epidermal growth factor (23). Activation of the phosphatidylinositol 3-kinase/AKT/FRAP pathway, which mediates signals from a broad range of growth factors, has likewise been demonstrated to increase HIF-1 α expression (24). Though the precise mechanism of these interfaces with the hypoxia-sensitive pathway is still not clear, those findings suggest a more general influence of growth-promoting stimuli on HIF-1 α activity. Thus, our results indicate that there may exist a different molecular event in tumor progression between SCCs and ACs.

Although the HIF-1 α protein is commonly up-regulated in a variety of cancers, the positive staining is known not to be universal. At present there is no certain explanation for this discrepancy. However, the prolonged fixation on pellets of hypoxic cells is known to substantially compromise antigen detection, so that failure to stain some tumors might be artificial. In the survey of tissue culture cells by immunoblot analysis, Wiesener et al. (4) found that under maximal hypoxic stimulation, all cells had detectable levels of at least one HIF α subunit, albeit the levels were quite variable. On the basis of the above finding, it is possible that relatively low levels of induced expression were still below the detection threshold in this immunohistochemical analysis, or that some tumors were relatively well oxygenated so that the HIF-1 α protein was not induced in the sections examined. Some tumors that were negative for the HIF-1 α protein in our cases also may express the HIF-1 α protein at levels below the limits of detection by the current immunohistochemical methodology. Otherwise other transcription factors that may have similar biological properties to HIF-1 α , such as HIF-2 α or HIF-3 α , may also mediate hypoxic adaptation in tumors (25, 26).

Zhong et al. (12) found that the HIF-1 α expression was noted in premalignant lesions such as colonic adenoma, breast ductal carcinoma in situ, and prostatic intraepithelial neoplasia, whereas every benign tumor was negative for the HIF-1 α expression. They suggested that HIF-1 α expression can occur very early in carcinogenesis. In our study, premalignant lesions were not included, but exceptionally a few of non-neoplastic epithelial cells adjacent to cancer showed the immunoreactivity for the HIF-1 α protein. Zhong et al. (12) also found a significant association of HIF-1 α expression with Ki-67 proliferation index. But we could not confirm a significant association of HIF-1 α expression with the PCNA index in our cases of SCC and AC, although the use of a different proliferation marker might be related with this discrepancy. We also found

that the PCNA index in overall SCCs was significantly higher than that in overall ACs, but within the category of HIF-1 α positive carcinomas the index did not show a significant difference between the two histologic types. Maybe this inconsistency stems from the hypothesis that pulmonary SCCs form a heterogeneous group of tumors with different biological properties and clinical behaviors (27). Feldser et al. (23) reported that the treatment of cultured prostatic carcinoma cells with insulin, IGF-1, or IGF-2 induced the expression of HIF-1 α protein, which was in turn required for expression of IGF-2 mRNA, suggesting the involvement of HIF-1 α protein in an autocrine growth factor loop. Thus, the HIF-1 α expression may be associated with growth factors, which endowed tumors with a higher PCNA index.

Since HIF-1 α stabilization up-regulates the expression of angiogenic and glycolytic pathways to restore oxygen homeostasis, the HIF-1 α protein may have an important role for the survival and growth of cancer. We examined the expression of the most representative angiogenic factor VEGF and MVD in SCCs and ACs. The MVD of SCCs was significantly higher than that of ACs, whereas the VEGF expression of SCCs showed no significant difference from that of ACs. Even within the category of HIF-1 α positive carcinomas, this tendency was also observed between SCCs and ACs. Thus, the MVD that we assessed by CD34 immunoreactivity was related to a specific histologic type of HIF-1 α positive carcinomas. These findings somewhat reflect the impact of HIF-1 α protein on the angiogenic process of common NSCLCs, especially SCCs. Otherwise the tendency of increasing MVD in SCCs could be associated with tumor necrosis, which was more frequently found in these types of cancer. On the contrary, Tsoi et al. (28), who used CD31 as the endothelial cell marker, reported no relationship between histologic types and MVD. This discrepancy is most likely associated with differences in the evaluation of the results, the use of different endothelial cell marker, and tumor heterogeneity. As concerned with VEGF, the lack of direct correlation between HIF-1 α and VEGF expression might suggest that although hypoxia triggers the expression of VEGF through HIF-1 α stabilization, the process of angiogenesis in pulmonary SCCs and ACs is also subject to other modulators such as platelet-derived endothelial cell growth factor, bcl-2, c-erbB-2, and MUC1 glycoprotein (29-31). But our result was in contrast with those of Giatromanolaki et al. (13), who reported the strong association between HIF-1 α expression and VEGF expression. This discordance may be related with the different interpretation methods in immunohistochemical staining. They regarded both nuclear and cytoplasmic staining as HIF-1 α positivity. As far as we know, although the HIF-1 α protein is synthesized in the cytoplasm and also degraded in the cytoplasm, it would be assumed that only nuclear HIF-1 α is the active form (15). However, we consider that the relationship between HIF-1 α and angiogenic factors needs to be clarified, with the development of more strict criteria

for immunohistochemical interpretation.

Finally, we observed no association between the overall survival of the patients and HIF-1 α expression ($p=0.442$). This result was rather different from that of Giatromanolaki et al. (13) who reported that HIF-1 α positive group was marginally related ($p=0.08$) to poor outcome. In addition, we also found no association between the overall survival and histologic parameters including histologic types, VEGF expression, MVD, and PCNA index. As concerned with MVD and VEGF expression, some workers suggested that the two parameters were not correlated with the survival (28, 32). However, other researchers reported that both MVD and VEGF expression were linked to poor survival (29, 33, 34). These discrepant observations may be explained by the different methodologies applied, the different approaches to the estimated results, and the cut-off levels used (28).

In conclusion, we suggest that the HIF-1 α expression, which can occur with hypoxic tumor environment in SCCs or with histological dedifferentiation in ACs, may play a differential role according to the histologic types of common NSCLCs. Thus, it seems that the process of HIF-1 α expression is controlled by different mechanisms between the two histologic types.

RERERENCES

1. Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT. *Reactive oxygen species generated at mitochondrial complex II stabilizes HIF-1 α during hypoxia: a mechanism of O₂ sensing.* *J Biol Chem* 2000; 275: 25130-8.
2. Ratcliffe PJ, Ebert BL, Firth JD, Gleadle JM, Maxwell PH, Nagao M, O'Rourke JF, Pugh CW, Wood SM. *Oxygen regulated gene expression: erythropoietin as a model system.* *Kidney Int* 1997; 51: 514-26.
3. Wenger PH. *Mammalian oxygen sensing, signaling and gene regulation.* *J Exp Biol* 2000; 203: 1253-63.
4. Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, Ratcliffe PJ, Maxwell PH. *Induction of endothelial PAS protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1 α .* *Blood* 1998; 92: 2260-8.
5. Wang GL, Jiang BH, Rue EA, Semenza GL. *Hypoxia-inducible factor-1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension.* *Proc Natl Acad Sci USA* 1995; 92: 5510-4.
6. Salceda S, Caro J. *Hypoxia-inducible factor 1 α (HIF-1 α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes.* *J Biol Chem* 1997; 272: 22642-7.
7. Huang LE, Gu J, Schau M, Bunn HF. *Regulation of hypoxia-inducible factor 1 α is mediated by an O₂ dependent degradation domain via the ubiquitin-proteasome pathway.* *Proc Natl Acad Sci USA* 1998; 95: 7987-92.
8. Hockel M, Schlenger K, Hockel S, Aral B, Schaffer U, Vaupel P. *Tumor*

- hypoxia in pelvic recurrences of cervical cancer. *Int J Cancer* 1998; 79: 365-9.
9. Stadler P, Becker A, Feldmann HJ, Hansgen G, Dunst J, Wurschmidt F, Molls M. Influence of the hypoxic subvolume on the survival of patients with head and neck cancer. *Int J Radiat Oncol Biol Phys* 1999; 44: 749-54.
 10. Dachs GU, Patterson AV, Firth JD, Ratcliffe PJ, Townsend KM, Stratford IJ, Harris AL. Targeting gene expression to hypoxic tumor cells. *Nat Med* 1997; 3: 515-20.
 11. Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, Ratcliffe PJ. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc Natl Acad Sci USA* 1997; 94: 8104-9.
 12. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW. Overexpression of hypoxia-inducible factor 1 α in common human cancers and their metastases. *Cancer Res* 1999; 59: 5830-5.
 13. Giatromanolaki A, Koukourakis MI, Sivridis E, Turley H, Talks K, Pezzella F, Gatter KC, Harris AL. Relation of hypoxia inducible factor 1 α and 2 α in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. *Br J Cancer* 2001; 85: 881-90.
 14. Travis WD, Colby TV, Corrin B, Shimosato Y, Brambilla E, Collaborators from 14 countries. World Health Organization Pathology Panel: World Health Organization. *Histological Typing of Lung and Pleural Tumors. International Histological Classification of Tumors. Third ed. Springer Verlag, Berlin, 1999.*
 15. Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, Pinedo HM, Abeloff MD, Simons JW, van Diest PJ, van der Wall E. Levels of hypoxia-inducible factor-1 α during breast carcinogenesis. *J Natl Cancer Inst* 2001; 93: 309-14.
 16. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. *N Eng J Med* 1991; 324: 1-8.
 17. Mattern J, Koomagi R, Volm M. Association of vascular endothelial growth factor expression with intratumoral microvessel density and tumour cell proliferation in human epidermoid lung carcinoma. *Br J Cancer* 1996; 73: 931-4.
 18. Semenza GL. Regulation of mammalian O₂ homeostasis by hypoxia-inducible factor 1. *J Biol Chem* 1995; 270: 1230-7.
 19. Chun YS, Kim MS, Park JW. Oxygen-dependent and -independent regulation of HIF-1 α . *J Korean Med Sci* 2002; 17: 581-8.
 20. Jiang BH, Agani F, Passaniti A, Semenza GL. V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. *Cancer Res* 1997; 57: 5328-35.
 21. Baas IO, Mulder JW, Offerhaus GJ, Vogelstein B, Hamilton SR. An evaluation of six antibodies for immunohistochemistry of mutant p53 gene products in archival colorectal neoplasms. *J Pathol* 1994; 172: 5-12.
 22. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumor suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature (Lond)* 1999; 399: 271-5.
 23. Feldser D, Agani F, Iyer NV, Pak B, Ferreira G, Semenza GL. Reciprocal positive regulation of hypoxia-inducible factor 1 α and insulin-like growth factor 2. *Cancer Res* 1999; 59: 3915-8.
 24. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, Semenza GL. Modulation of hypoxia-inducible factor 1 α expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/ AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res* 2000; 60: 1541-5.
 25. Hogenesch JB, Chan WK, Jackiw VH, Brown RC, Gu YZ, Pray-Grant M, Perdew GH, Bradfield CA. Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. *J Biol Chem* 1997; 272: 8581-93.
 26. Gu YZ, Moran SM, Hogenesch JB, Wartman L, Bradfield CA. Molecular characterization and chromosomal localization of a third α -class hypoxia inducible factor subunit, HIF3 α . *Gene Expr* 1998; 7: 205-3.
 27. Mattern J, Koomagi R, Volm M. Biological characterization of subgroups of squamous cell lung carcinomas. *Clin Cancer Res* 1999; 5: 1459-63.
 28. Tsoi E, Zacharatos P, Dasiou-Plakida D, Peros J, Evangelou K, Zavras AI, Yannoukakos D, Konstantopoulou I, Asimacopoulos PJ, Kittas C, Gorgoulis VG. Growth index is independent of microvessel density in non-small-cell lung carcinomas. *Hum Pathol* 2002; 33: 812-8.
 29. O'Byrne KJ, Koukourakis MI, Giatromanolaki A, Cox G, Turley H, Steward WP, Gatter K, Harris AL. Vascular endothelial growth factor, platelet-derived endothelial cell growth factor and angiogenesis in non-small-cell lung cancer. *Br J Cancer* 2000; 82: 1427-32.
 30. Koukourakis MI, Giatromanolaki A, O'Byrne KJ, Cox J, Krammer B, Gatter KC, Harris AL. bcl-2 and c-erbB-2 proteins are involved in the regulation of VEGF and of thymidine phosphorylase angiogenic activity in non-small cell lung cancer. *Clin Exp Metastasis* 2000; 17: 545-54.
 31. Giatromanolaki A, Koukourakis MI, Sivridis E, O'Byrne K, Cox G, Thorpe PE, Gatter KC, Harris AL. Co-expression of MUC1 glycoprotein with multiple angiogenic factors in non-small cell lung cancer suggests co-activation of angiogenic and migratory pathways. *Clin Cancer Res* 2000; 6: 1917-21.
 32. Decaussin M, Sartelet H, Robert C, Moro D, Claraz C, Brambilla C, Brambilla E. Expression of vascular endothelial growth factor (VEGF) and its two receptors (VEGF-R1-Flt1 and VEGF-R2-Flk1/KDR) in non-small cell lung carcinomas (NSCLCs): correlation with angiogenesis and survival. *J Pathol* 1999; 188: 369-77.
 33. Giatromanolaki A, Koukourakis MI, Kakolyris S, Turley H, O'Byrne K, Scott PA, Pezzella F, Georgoulis V, Harris AL, Gatter KC. Vascular endothelial growth factor, wild-type p53, and angiogenesis in early operable non-small cell lung cancer. *Clin Cancer Res* 1998; 4: 3017-24.
 34. Fontanini G, Vignati S, Boldrini L, Chine S, Silvestri V, Lucchi M, Mussi A, Angeletti CA, Bevilacqua G. Vascular endothelial growth factor is associated with neovascularization and influences progression of non-small cell lung carcinoma. *Clin Cancer Res* 1997; 3: 861-5.