

**DNA-damage inducible 1 is a  
property of human non-small cell  
lung cancer**

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**DNA-damage inducible 1 is a  
property of human non-small cell  
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Directed by Professor Se Kyu Kim

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Ji Yeon Lee

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This certifies that the Master's Thesis of  
Ji Yeon Lee is approved.

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<ABSTRACT>

**DNA-damage inducible 1 is a property of human non-small cell lung cancer**

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**Background :** DNA damage-inducible 1 (Ddi1), one of the ubiquitin-like and ubiquitin-associated family of proteins, may function in the regulation of the ubiquitin–proteasome pathway, which has been validated as a target for antineoplastic therapy.

**Objective :** We investigated Ddi1 expression in human lung cancer tissues and evaluated the relationship of this expression pattern with clinicopathological factors in patients with non-small-cell lung cancer (NSCLC).

**Methods :** Ddi1 expression was examined by immunohistochemistry in the tumor tissues from 97 patients with stage I NSCLC, who had undergone curative surgical resection at two tertiary referral hospitals from 1993–2004. None of the patients received preoperative chemotherapy and/or radiation therapy.

**Results :** Thirty-nine (40.2%) of the 97 cases were positive for Ddi1. Ddi1 expression was seen dominantly in cytoplasm rather than in the nuclei of cancer cells in all histological types, whereas adjacent nontumoral lung tissue showed negative Ddi1 staining in most cases. Ddi1 expression tended to increase in

well-differentiated tumors but without statistical significance. Positive Ddi1 expression was associated with a tendency for better disease-free survival and disease-specific survival, although the difference was not significant.

**Conclusions** : Ddi1 expression is a property of NSCLC. Because Ddi1 could be a potential target for cancer therapy, more work is needed to evaluate its role in NSCLC.

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Key words : DNA damage-inducible 1, lung cancer, immunohistochemistry

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## I. INTRODUCTION

Lung cancer is one of the most commonly diagnosed cancers as well as the leading cause of cancer deaths worldwide.<sup>1</sup> However, only about 20% of patients with non-small cell lung cancer (NSCLC) have their disease diagnosed at a stage in which surgery can be recommended as part of initial care. Moreover, the 5-year survival rate of even the earliest stage I lung cancers after complete surgical resection is still only approximately 70%, leading to the need for novel therapeutic targets and modalities.<sup>2</sup>

One of the strategies for lung cancer therapy is targeting the ubiquitin-proteasome system (UPS), which plays a central role in cell homeostasis, resulting in deregulation of cell processes necessary for survival.<sup>3, 4</sup> DNA damage-inducible 1 (Ddi1) belongs to the ubiquitin-like (UbL) and ubiquitin-associated (UBA) family of proteins that has been implicated in the regulation

of UPS.<sup>5</sup> Ddi1 appears to release substrate from an ubiquitination complex, making the substrate available for deubiquitination, and, thus, is presumed to play an important role facilitating proteasomal degradation. Additionally, Ddi1 is the only representative in which the UbL and UBA domains flank an aspartyl protease-like (RVP) domain. This central Ddi1 domain has a remarkable structural similarity with retroviral proteases, suggesting that Ddi1 functions proteolytically during regulated protein turnover in the cell. Notably, current studies show that this RVP domain is a potential therapeutic target for retroviral aspartic protease inhibitors.<sup>6</sup>

Much effort has been made to reposition the established retroviral aspartic protease inhibitors as anticancer agents for patients with NSCLC. Recent studies have shown that nelfinavir, ritonavir, and saquinavir inhibit the growth of NSCLC and every cell type among 60 kinds of cancer cells<sup>7</sup>. Nelfinavir was the most effective of all protease inhibitors tested, resulting in apoptosis and non-apoptotic cell death. Non-apoptotic cell death was related to induction of endoplasmic reticulum stress, which subsequently led to autophagy, a normal process of self digestion that generates energy for the cell under conditions of stress<sup>8</sup>. Consequentially, these results suggest that nelfinavir could be repositioned as a lung cancer therapeutic, and one of the potential targets for this promising drug is Ddi1.

However, the clinical role of Ddi1 in human cancer is unknown. Therefore, in

this study, we investigated Ddi1 immunohistochemical expression in human lung cancer tissue and evaluated whether Ddi1 expression increased in lung cancer compared with that in adjacent nontumoral tissue. We also investigated Ddi1 function by assessing intracellular localization as well as tissue distribution of Ddi1 expression to confirm whether Ddi1 could be developed as a useful target for nelfinavir.

## II. MATERIALS AND METHODS

### 1. Study Subjects

The study group comprised 97 patients with stage I NSCLC. Patients underwent curative surgical resection at two tertiary referral hospitals (Severance Hospital and Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea) from 1993–2004. None of the patients received preoperative chemotherapy and/or radiation therapy.

Patient charts, including pathology and operative reports, were reviewed, and the data were coded. One representative formalin-fixed paraffin-embedded primary tumor block was obtained for each case.

This study was approved by the Institutional Review Board of Severance Hospital (4-2011-0409). The board waived informed consent from the patients. This study was conducted in accordance with the Declaration of Helsinki.

### 2. Immunohistochemistry (IHC)

Sections (4- $\mu$ m thick) were prepared from each of the paraffin-embedded samples. After sections were deparaffinized in xylene (three 10-minute washes) and dehydrated in a graded ethanol series (100, 90, 70, and 50%, 5-minute washes), endogenous peroxidase activity was blocked with 30% hydrogen peroxide in water for 5 min. Antigens were retrieved for Ddi1 staining by boiling in 10 mM citrate acid (pH 6.0) for 3 min using a microwave oven. Sections were then incubated with rabbit anti-Ddi1 polyclonal antibodies

(GeneTex, Irvine, CA, USA) and diluted 1:100 in antibody diluents (DAKO, Glostrup, Denmark) for 1 hr at 37°C. Secondary biotinylated link anti-mouse IgG or anti-rabbit IgG for the Labeled Streptavidin-Biotin2 System, horseradish peroxidase (DAKO), and a third streptavidin peroxidase conjugate (DAKO) were incubated with the sections at room temperature. The reaction was visualized with the 3,3-diaminobenzidine substrate system (DAKO). Hematoxylin was used as the counterstain. Human placenta tissue was used as a positive control because of its easy availability and relatively stable reactivity. The negative control consisted of isotype control antibody (rabbit polyclonal IgG, DAKO) substituted for the primary Ddi1 antibody. Controls were run with each batch of slides at an average of approximately 15 slides per batch.

### 3. IHC Interpretation

Slides were interpreted by one experienced pathologist (LBJ). The intensity of Ddi1 expression as evaluated microscopically was graded on a scale of 0–3+ with 3 being the highest expression observed (0, no staining; 1+, weak; 2+, moderate; 3+, intense). Staining density was quantified as the percentage of cells staining positive for Ddi1 with the primary antibody as follows: 0, no staining; 1, positive staining in <25% of the sample; 2, positive staining in 25–50% of the sample; 3, positive staining in >50% of the sample. Intensity score (0–3+) was multiplied by the density score (0–3) to give an overall score of 0–9.

The overall score for each specimen was then categorically assigned to one of the following groups: 0, negative expression; 1–2, weak expression; 3–6, moderate expression; 9, strong expression.

#### 4. Statistical analyses

Continuous variables were analyzed using nonparametric tests (Mann–Whitney *U*-test or Kruskal–Wallis test), and categorical variables were compared with the Pearson’s  $\chi^2$  or Fisher’s exact tests, as appropriate. Survival curves and recurrence-free survival curves were estimated using the Kaplan–Meier method, and statistical significance between survival curves was assessed by the log-rank test. Multivariate analysis was performed using Cox’s proportional hazards regression model. SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for all analyses. A *p*-value < 0.05 was considered significant.

### III. RESULTS

#### 1. Clinicopathological variables

The demographic and clinical characteristics of the enrolled patients are listed in Table 1. Age, gender, and histological distributions in our population agreed with the literature for stage I NSCLC<sup>9, 10</sup>. The median age of the patients was 64 years (range 32–83 years), and the majority of patients were male (78.4%). The NSCLC tumors were comprised of 48 squamous cell carcinomas, 45 adenocarcinomas, three large-cell carcinomas, and one adenosquamous cell carcinoma. Twenty-three (23.7%) patients were stage IA and 74 (76.3%) were stage IB.

**Table 1. Clinicopathological features related to DNA damage-inducible 1 (Ddi1) expression in non-small-cell lung cancer (NSCLC)**

	Total	Ddi1 Expression		<i>p</i> -value*
		Negative	Positive	
No. (%)	97 (100%)	58 (59.8%)	39 (40.2%)	
Age, median (range), years	64 (32-83)	62.5 (32-83)	64 (32-77)	0.897 <sup>†</sup>
Male sex, n (%)	76 (78.4%)	46 (60.5%)	30 (39.5%)	0.780
Ever smoker	47 (48.5%)	26 (55.3%)	21 (44.7%)	0.557
Pathological stage, n (%)				
IA	23 (23.7%)	14 (60.9%)	9 (39.1%)	> 0.999
IB	74 (76.3%)	44 (59.5%)	30 (40.5%)	
Pathology				
Squamous cell carcinoma	48 (49.5%)	27 (56.3%)	21 (43.8%)	0.915
Adenocarcinoma	45 (46.4%)	28 (62.2%)	17 (37.8%)	
Large cell carcinoma	3 (3.1%)	2 (66.7%)	1 (33.3%)	
Adenosquamous cell carcinoma	1 (1.0%)	1 (100%)	0 (0%)	
Histologic differentiation				
Well	25 (31.3%)	11 (44.0%)	14 (56.0%)	0.393
Moderate	31 (38.8%)	18 (58.1%)	13 (41.9%)	
Poor	22 (27.5%)	15 (68.2%)	7 (31.8%)	
Undifferentiate	2 (2.5%)	1 (50.0%)	1 (50.0%)	

\* Pearson's  $\chi^2$  or Fisher's exact test were used to estimate *p*-values.

<sup>†</sup> Mann–Whitney *U* test was used to estimate the *p*-value.

## 2. Ddi1 is mainly localized in the cytoplasm of NSCLC cells

Figure 1 shows IHC staining patterns for the positive and negative controls. Ddi1 expression was seen in the cytoplasm of most syncytiotrophoblasts in normal placenta, which served as the positive control.

Of the 97 NSCLC cases, 39 (40.2%) were interpreted as Ddi1 positive, including 21 (43.8%) of 48 squamous cell carcinomas, 17 (37.8%) of 45 adenocarcinomas, and one of three large cell carcinomas (Table 1). Ddi1 expression was localized to the tumor cells, whereas bronchial epithelial cells, pneumocytes, and chondrocytes from bronchial cartilage in the adjacent nontumoral lung tissue showed Ddi1 negative staining in most cases examined. In all of the positive cases, Ddi1 expression was seen dominantly in the cytoplasm of cancer cells rather than in the nucleus, regardless of histological type. A cytoplasmic membrane staining pattern was observed in addition to the cytoplasmic and nuclear staining in one case of poorly differentiated adenocarcinoma (Fig. 1).

**Figure 1. DNA damage-inducible 1 (Ddi1) expression in controls and lung cancer tissues; positive control (A), negative control (B), bronchioalveolar carcinoma (C), adenocarcinoma (D), squamous cell carcinoma (E), large cell carcinoma (F), and adjacent nontumoral lung tissue (G) (×200).**

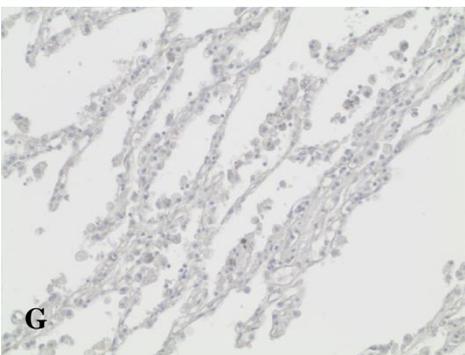
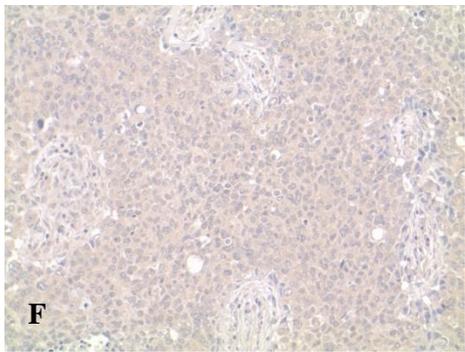
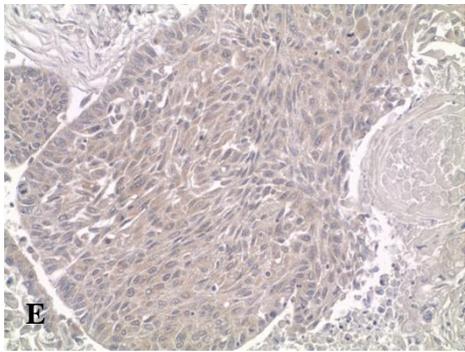
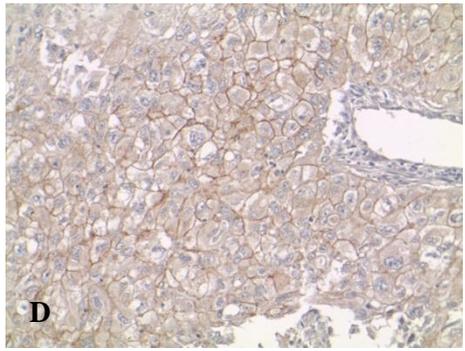
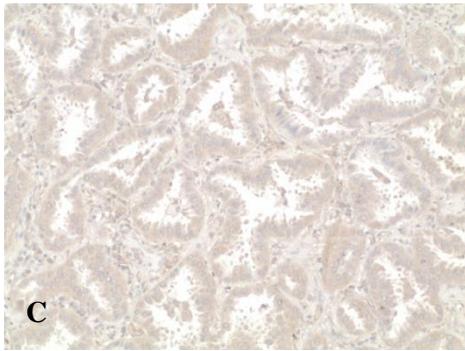
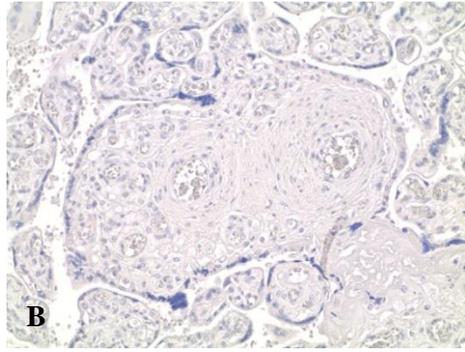
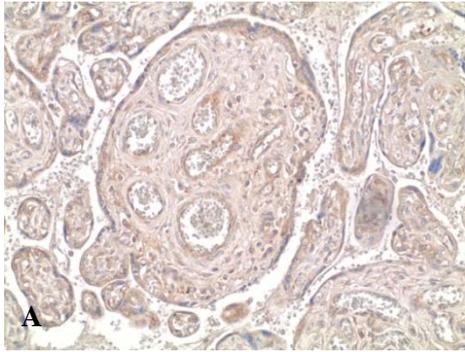


Table 1 compares various parameters between the Ddi1 negative and positive groups. Histological subtype was not related to Ddi1 expression. Age, gender, smoking history, and pathological stage also did not differ between the groups. Ddi1 expression tended to increase with NSCLC differentiation; however, the difference was not significant. Table 2 shows Ddi1 expression according to histological subtype. No differences in age, pathological stage, or histological differentiation were observed between the Ddi1 expression groups when subgroup analyses were performed for the histological subtypes (Table 3).

**Table 2. DNA damage-inducible 1 (Ddi1) expression according to non-small-cell lung cancer (NSCLC) histological subtype**

	Total (N=97)	Squamous cell (n=48)	Adenoca (n=45)	Large cell (n=3)
<b>Scores</b>				
0	58 (59.8%)	27 (56.3%)	28 (62.2%)	2 (66.7%)
1	8 (8.2%)	5 (10.4%)	3 (6.7%)	0 (0%)
2	8 (8.2%)	7 (14.6%)	1 (2.2%)	0 (0%)
3	12 (12.4%)	5 (10.4%)	6 (13.3%)	1 (33.3%)
4	5 (5.2%)	3 (6.3%)	2 (4.4%)	0 (0%)
6	4 (4.1%)	0 (0%)	4 (8.9%)	0 (0%)
9	2 (2.1%)	1 (2.1%)	1 (2.2%)	0 (0%)
<b>Expression</b>				
Weak	16 (16.5%)	12 (25.0%)	4 (8.9%)	0 (0%)
Moderate	21 (21.6%)	8 (16.7%)	12 (26.7%)	1 (33.3%)
Strong	2 (2.1%)	1 (2.1%)	1 (2.2%)	0 (0%)

**Table 3. Clinicopathological features related to DNA damage-inducible 1 (Ddi1) expression in histologic subtypes of non-small-cell lung cancer (NSCLC)**

	Squamous cell carcinoma			Adenocarcinoma		
	Ddi1 expression		<i>p</i> -value*	Ddi1 expression		<i>p</i> -value*
	Negative	Positive		Negative	Positive	
Age, median (range), years	65 (48-78)	68 (51-77)	0.244 <sup>†</sup>	59 (32-83)	57 (32-73)	0.690 <sup>†</sup>
Male sex, n (%)	23 (85.2%)	20 (95.2%)	0.369	20 (69.0%)	9 (31.0%)	0.209
Ever smoker	16 (94.1%)	17 (94.4%)	> 0.999	9 (75.0%)	3 (25.0%)	0.129
Pathological stage, n (%)						
IA	5 (18.5%)	2 (9.5%)	0.445	9 (56.3%)	7 (43.8%)	0.539
IB	22 (81.5%)	19 (90.5%)		19 (65.5%)	10 (34.5%)	
Histologic differentiation						
Well	3 (11.5%)	6 (30.0%)	0.240	11 (55.0%)	9 (45.0%)	0.900
Moderate	14 (53.8%)	10 (50.0%)		4 (57.1%)	3 (42.9%)	
Poor	9 (34.9%)	4 (20.0%)		6 (66.7%)	3 (33.3%)	

\* Pearson's  $\chi^2$  or Fisher's exact test were used to estimate *p*-values.

<sup>†</sup> Mann–Whitney *U* test was used to estimate the *p*-value.

### 3. Clinical and pathological correlations

The median follow-up duration was 120 months. In a univariate analysis, the positive Ddi1 expression group tended to show lower rates of tumor recurrence,

distant metastasis, cancer-related death, and all-cause mortality but without statistical significance (Table 4).

**Table 4. Correlations between DNA damage-inducible 1 (Ddi1) expression and clinical outcome**

	Ddi1 Expression		<i>p</i> -value*
	Negative	Positive	
Recurrence	23 (39.7%)	12 (30.8%)	0.372
Local recurrence	9 (39.1%)	8 (66.7%)	0.122
Distant metastasis	14 (60.9%)	4 (33.3%)	
Cancer-related death	16 (27.6%)	9 (23.1%)	0.619
All-cause mortality	26 (44.8%)	16 (43.2%)	0.879

\* Pearson's  $\chi^2$  and Fisher's exact test were used to estimate *p*- values.

Of 97 patients, 12 (30.8%) of 39 Ddi1 positive patients and 23 (39.7%) of 58 Ddi1 negative patients experienced a recurrence. The Kaplan–Meier disease-free survival (DFS) estimates for patients grouped according to Ddi1 expression are shown in Fig. 2-A; the median DFS time was 168.23 months in the Ddi1 positive group but was not reached in the Ddi1 negative group. The Ddi1 positive group tended to have a higher 5-year DFS rate than that in the Ddi1 negative group but the difference was not significant (79.2% vs. 72.3%).

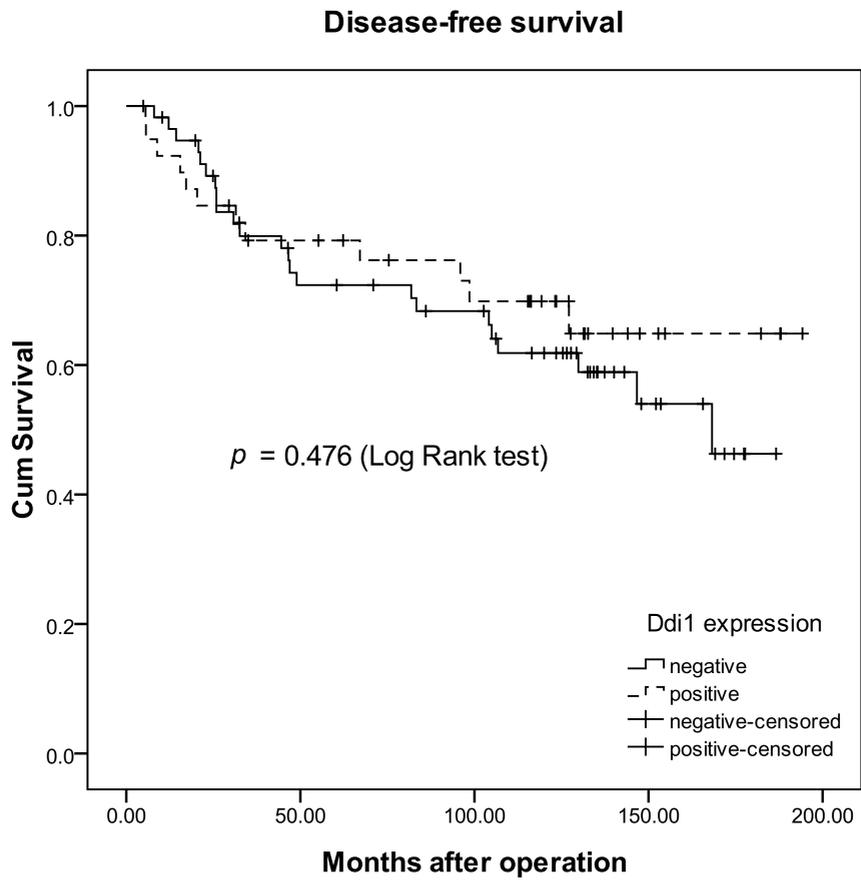
Nine (23.1%) and 16 (27.6%) cancer-related deaths occurred in Ddi1 positive and negative patients, respectively. The median disease-specific survival (DSS) time was not reached in either Ddi1 expression group, whereas the estimated 5-year DSS rate was the same (81.4%) (Fig. 2-B).

The median overall survival (OS) times were 152.80 and 174.40 months, and the estimated 5-year OS rates were 73.0% and 74.1% in the Ddi1 positive and negative groups. The Kaplan–Meier estimates for both groups showed no significant difference in OS (Fig. 2-C). The Cox regression analysis adjusting for pathological stage and age demonstrated no significant difference in DFS, DSS, or OS between the Ddi1 expression groups (data not shown).

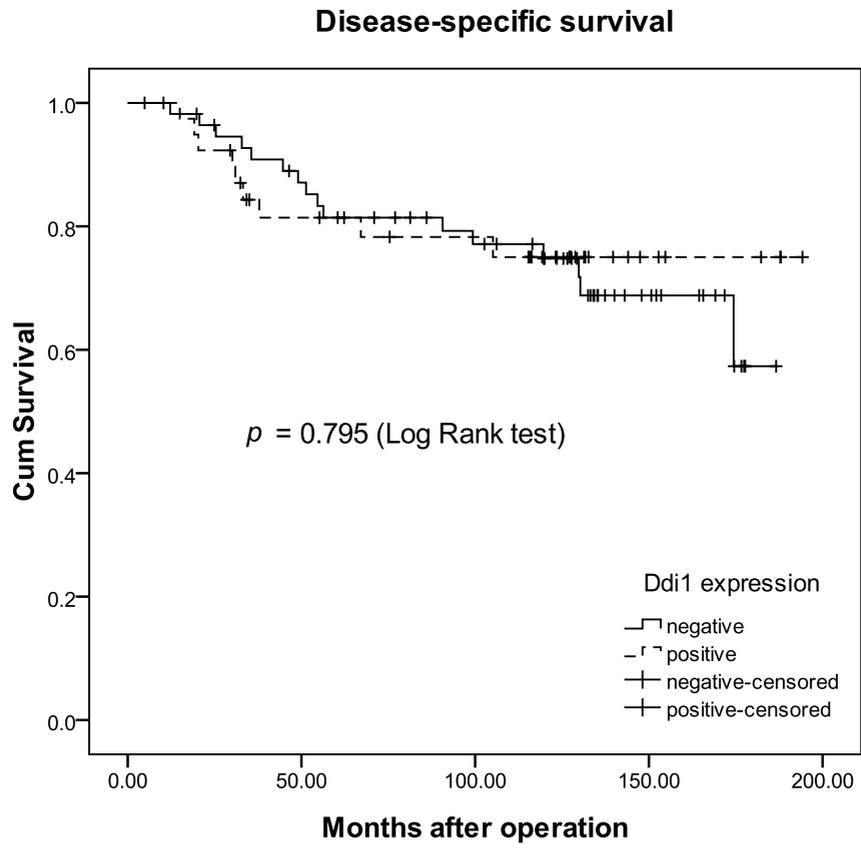
The results were similar when subgroup analyses were performed for the histological subtypes. Ddi1 expression did not significantly influence DFS, DSS, or OS of patients with squamous cell carcinoma or adenocarcinoma (data not shown).

**Figure 2. Survival analysis of patients with stage I non-small-cell lung cancer stratified for DNA damage-inducible 1 (Ddi1) expression; Disease free survival (A), disease specific survival (B) and overall survival (C) of patients with non-small-cell lung cancer.**

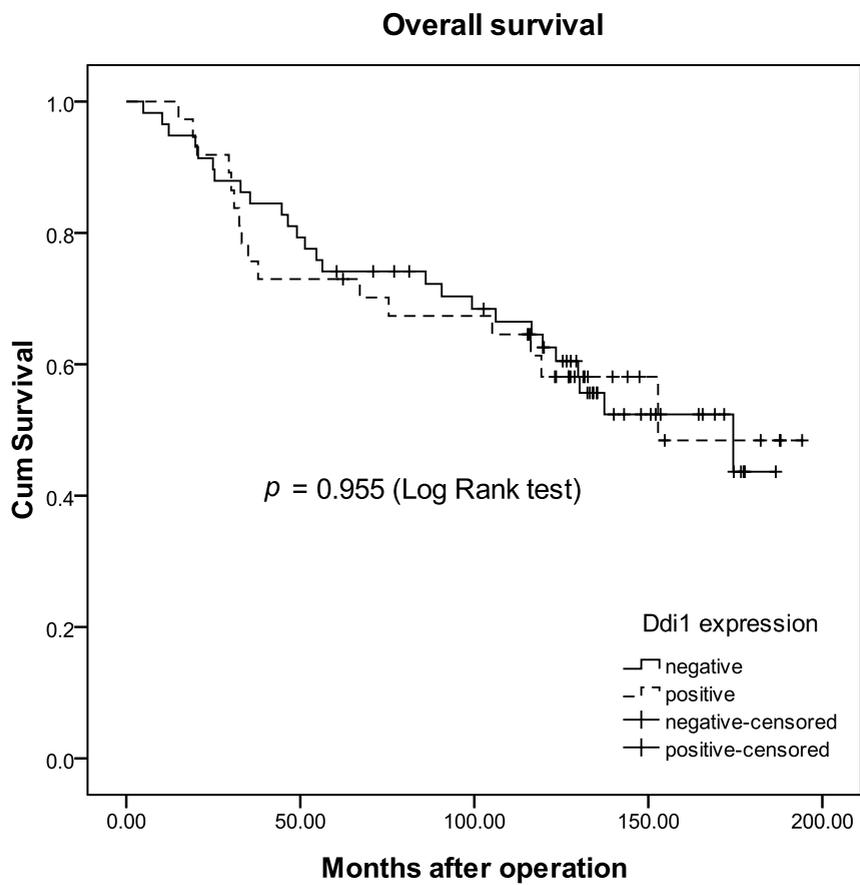
A.



**B.**



C.



#### IV. DISCUSSION

Among the UbL-UBA family of proteins, Ddi1 is the least studied and has never been associated with lung cancer. In the present study, IHC showed positive Ddi1 staining in about 40% of lung cancer tissues, and the proportions were similar among histological subtypes. Among the positive cases, most of the adjacent nontumoral lung tissue was negative for Ddi1 expression, whereas cancer cells showed diffuse staining for Ddi1, suggesting that Ddi1 could be a potential selective target for lung cancer treatment.

Previous studies have shown that Ddi1 localizes preferentially to the nucleus in wild-type cells, which could imply its function in nuclear-associated ubiquitin-dependent proteolysis<sup>11, 12</sup>. Unexpectedly, we found that Ddi1 expression occurred not only in the nucleus, but also in the cytoplasm, even in the cell membranes of cancer cells. Therefore, we postulate that any alteration of the role of Ddi1 in the interaction with cell cycle-related proteins and mediating their stability may have influenced Ddi1 localization in NSCLC cells.

The role of Ddi1 in targeting proteins for proteasomal degradation is well established. A loss of Ddi1 results in stabilization of ubiquitinated substrates, which accumulate in the cytoplasm. The substrate can only interact with the proteasome in the presence of Ddi1 when it is ubiquitinated, suggesting that the

initial interaction must be between a ubiquitin chain and the Ddi1 protein.<sup>11</sup> Because ubiquitination is critical for proliferation and survival of cancer cells, Ddi1 dysregulation may result in cancer by creating a UPS imbalance.

In the present study, poor differentiation of tumor cells tended to be correlated with negative Ddi1 expression, but the correlation was not significant. Different results have been reported on the relationship between tumor differentiation and expression of UPP components. Some studies have reported that cancers and rapidly growing embryonic cells generally have higher levels of proteasome activity than their normal well-differentiated counterparts,<sup>13, 14</sup> and others have found that Ub levels increase during development and differentiation<sup>15, 16</sup>. In contrast, another study showed no relationship between ubiquitin levels and the degree of histological differentiation<sup>17</sup>. The present results showed that positive Ddi1 expression was associated with well-differentiated tumors, which behave in an indolent fashion. Thus, we presumed that Ddi1 might play a greater role during the initial stage of carcinogenesis than in the progressing stage when tumors develop more aggressive behavior, although it is difficult to assume the common roles of Ddi1 during oncogenesis. This possibility parallels our results showing a higher rate of local recurrence than distant metastasis in recurrent Ddi1 positive patients, whereas a higher rate of distant metastasis was observed in patients who were Ddi1 negative. Furthermore, Ddi1 expression was

associated with a more indolent clinical course with better disease-free and disease-specific survivals. However, these results did not reach statistical significance, probably because of the relatively small sample size; thus, further studies are needed to confirm or refute this association.

## V. CONCLUSION

In conclusion, we observed positive Ddi1 staining in about 40% of NSCLC tissues. Our results suggest that Ddi1 expression is a property of NSCLC that appears to be associated with a more indolent clinical course. Ddi1 could be a potential target for cancer therapy. Therefore, more work is needed to evaluate the role of Ddi1 in NSCLC.

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< ABSTRACT(IN KOREAN)>

제 1 병기 비소세포폐암 환자에서 DNA-damage inducible 1  
발현의 임상적 의의  
<지도교수 김 세 규>

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배경: DNA damage-inducible 1 (Ddi1) 은 ubiquitin-like and ubiquitin-associated family of protein 중의 하나로, 최근 항암치료의 표적으로 주목받고 있는 유비퀴틴-프로테아좀 경로를 조절하는 기능을 하는 것으로 알려져 있다.

목적: 본 연구에서는 사람의 비소세포폐암 조직에서 Ddi1 의 발현 정도를 확인하고 임상 양상 및 병리학적 양상과의 연관성을 조사하고자 하였다.

방법: 연구대상은 1993년부터 2004년까지 서울 세브란스병원 및 강남세브란스병원에서 근치적 절제술을 시행 받은 제 1기 비소세포폐암 환자들을 포함하였으며 수술 전 보조항암요법 및 방사선요법을 받은 환자는 제외하였다. 환자 폐암 조직에서 면역조직화학염색을 시행하여 Ddi1의 발현 정도를 평가하였다.

결과: 총 97례 중 39례(40.2%)에서 Ddi1 양성으로 판정되었다. 모든 조직학적 유형에서 Ddi1의 발현은 주로 암세포의 세포질에서 관찰된

반면 주변 정상양 폐조직에서는 Ddi1의 발현이 관찰되지 않았다. Ddi1의 발현은 암의 조직학적 분화도가 좋을수록 증가하는 경향을 보였으며 Ddi1 양성인 환자들의 경우 무병 생존률과 질병 특이 생존률이 증가하는 소견이 관찰되었으나 통계적인 유의성은 보이지 않았다. 결론: Ddi1의 발현은 비소세포폐암의 특성 중 하나이다. Ddi1은 유비퀴틴-프로테아좀 경로를 표적으로 하는 항암제의 목적 물질로 개발될 가능성이 있으므로 비소세포폐암에서 Ddi1의 역할을 평가하기 위한 추가적인 연구가 필요할 것으로 사료된다.

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핵심되는 말 : DNA damage-inducible 1, 폐암, 면역조직화학