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Elevated Serum β-Defensins Concentrations in Patients with Lung Cancer

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Abstract. *Background: Beta-defensins (HBDs) are expressed in lung epithelial cells and act as antimicrobial agents. Most lung cancers that originate from pulmonary epithelial cells may produce HBDs. Materials and Methods: We measured serum HBD-1 and HBD-2 levels in healthy subjects (HS), patients with lung cancer and patients with pneumonia by radioimmunoassay. Results: Serum HBD-1 levels were higher in patients with lung cancer than HS and patients with pneumonia. Serum HBD-2 levels were higher in patients with lung cancer than HS. When cut-off values for positive HBD-1 were set at mean + 2SD of HS, the sensitivity and specificity of HBD-1 for the whole group of patients with lung cancer were 76.4 and 94.0 %, respectively, and the proportion of patients with HBD-1-positive lung cancer and clinical stage I was 69.2 %. Conclusion: Serum HBDs levels were high in patients with lung cancer and the serum HBD-1 level could be used as an auxiliary diagnostic tool for lung cancer.*

Lung cancer is the leading cause of cancer-related death in developed countries. Surgical resection represents the standard of care in early-stage lung cancer (1), though it remains difficult to detect early-stage lung cancer and to distinguish benign and malignant tumours, particularly when the size of the tumour is small. At present, tumour markers for lung cancer are useful for assessing the effects of treatment, but are not appropriate for detection of early-stage lung cancer because of their low positive rates (2,3). Tumour cells in lung cancer are known to produce various molecules, such as tumour growth-associated cytokines including interleukin (IL-8) and tumour growth factor (TGF)-α (4,5). Products released from tumour cells could be used as tumour markers if they could be detected in early-stage lung cancer.

Defensins are antimicrobial peptides produced by neutrophils and epithelial cells (6-8). These peptides provide non-specific host defence against micro-organisms. According to the alignment of the disulfide bridges, human defensins can be divided into two classes: α- and β-defensins (6-8). Six α-defensins and four β-defensins [(HBD)-1, 2, 3 and 4] have been identified in humans. Plasma concentrations of α-defensins and HBD-2 are elevated in patients with bacterial infections, but diminish during the recovery phase (9,10). Epithelial cells of the lungs and kidneys constitutively express HBD-1, whereas production of HBD-2 by epithelial cells of the lungs and skin is induced by contact with micro-organisms (11). Defensins are cytotoxic to tumour cells as well as micro-organisms (12-14) and have been recently reported to induce cytokine production (15). We have previously prepared an antiserum against HBD-1 and HBD-2, and established a sensitive and specific radioimmunoassay (RIA) for HBDs (10,16). The aims of the present study were to determine the serum concentrations of HBDs in patients with lung cancer, the correlate between their levels and lung cancer tumour type and growth, and the suitability of HBDs as markers of lung cancer.

Materials and Methods

Subjects. Fifty-six patients with lung cancer [31 adenocarcinomas (Ad), 9 small cell carcinomas (Sm), 15 squamous cell carcinomas (Sq) and 1 large cell carcinoma (Lg)] were recruited in the present study, representing all consecutive patients admitted to Miyazaki Medical College and Miyazaki Prefectual Hospital, Japan, with a diagnosis of lung cancer between January 2001 and January 2002. The group consisted of 15 women and 41 men with a mean age of 66.4±10.5 years (mean±SD) and included 36 smokers, 1 ex-smoker and 19 non-smokers. The clinical stage of cancer was determined according to the 1997 International System for Staging Lung Cancer, UICC (17), into stage I (n=13), II (n=2), IIIA (n=4), IIIB (n=16) and IV (n=21). Patients with infections based on cultures of sputum, blood and urine were excluded from the study. We also selected 18

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patients with bacterial pneumonia and 46 healthy volunteers as control. Patients with bacterial pneumonia comprised 6 women and 12 men with a mean age of 63.2±20.8 years. Bacterial pneumonia was diagnosed based on the following criteria: i) typical clinical features consistent with pneumonia (fever, cough, sputum), ii) infiltrates on chest X-ray, iii) peripheral neutrophilia, iv) identification of pathogen by sputum culture, and v) prompt response to antibiotic therapy. Healthy volunteers comprised 24 women and 22 men with a mean age of 33.1±9.7 years. Informed consent was obtained from all subjects at entry to this study.

Blood sampling. Blood samples (2 ml from each patient) were obtained from all patients before treatment. Three patients with lung cancer underwent lung resection with complete lymph nodes dissection, and blood was again obtained after surgical resection. The blood sample was centrifuged to obtain serum and 0.01 to 0.1mL serum samples were subjected to RIA for HBD-1 and -2.

Measurement of serum HBD concentrations. The concentrations of HBD-1 and -2 were measured by RIA established in our laboratory (10,16). HBDs were radioiodinated by the lactoperoxidase method, and ^{125}I-labelled peptide was purified by reverse phase high-performance liquid chromatography on a column (model TSK ODS 120A; Tosoh Co., Tokyo, Japan). The incubation buffer for the RIA was 50 mM sodium phosphate (pH 7.4) containing 0.25% bovine serum albumin treated with N-ethylmelemide, 80 mM NaCl, 25 mM ethylenediaminetetraacetic acid - 2Na, 0.05% NaN_3, 0.1% octoxynol-9 (Triton X-100), acid 3.1% dextran T-40. The diluted sample or a standard peptide solution (100 μL) was incubated for 24 h with 100 μL diluted antiserum (final dilutions, 1:460,000 and 1:420,000, respectively). The tracer solution (16,000 to 18,000 counts per min in 100 μL of solution) was added, and the mixture was incubated for 24 h, after which normal rabbit serum and anti-rabbit IgG goat serum were added and the whole preparation was stored for another 16 h. Bound and free ligands were separated by centrifugation. All procedures were performed at 4˚C and the samples were assayed in duplicate.

Measurement of various tumour markers in the serum. Serum concentrations of carcinoembryonic antigen (CEA) were measured in 52 patients with lung cancer by immunohistochemistry assay using ST AIA-PACK CEA (Tosoh Co.). Serum concentrations of squamous cells carcinoma antigen (SCC-Ag) and neuron-specific enolase (NSE) were determined in 48 and 44 patients with lung cancer, respectively, by immunoradiometric assay using SCC RIABEAD (Dainabot Co., Tokyo, Japan) and Ab BEAD NSE "EIKEN" (Eiken Chemical Co., Tokyo, Japan), respectively. The cut-off levels for CEA, SCC-Ag and NSE using lung cancer as positive were 4.7 ng/ml (mean of control subjects+2SD), 1.5 ng/ml (mean of control subjects+2SD) and 10 ng/ml (mean of control subjects+3SD), respectively.

Immunohistochemical staining for HBD-1 and -2 in lung tumours. Lung cancer specimens were obtained from 3 patients (Ad, Sq and Lg) by surgical resection. The tissue samples were fixed with Zamboni solution (2% paraformaldehyde and 0.25% picric acid in 0.1 M phosphate-buffered saline [PBS], adjusted to pH 7.4) or 10% formaldehyde in PBS. After dehydration in serial ethanol solutions, the tissue samples were embedded in paraffin. The specimens (3-mm-thick) were deparaffinised in xylene, rehydrated in serial ethanol solutions and treated with 0.3% hydrogen peroxide for 60 min to inactivate any endogenous peroxidase. Non-specific binding was blocked with normal goat serum. Anti-HBD (HBD-1 and -2) antiserum at a final dilution of 1/2000 was allowed to react overnight with each preparation at 4˚C in a moist chamber. Goat biotinylated anti-rabbit IgG was used as the second antibody. The samples were stained by ABC alkaline phosphatase (ABC-AP) method using an ABC-AP kit (Dako Co., Carpinteria, CA, USA) and the tissue samples were counterstained with haematoxylin.

Statistical analysis. Data were expressed as mean±SD. Differences between groups were examined using the analysis of variance (ANOVA) and Scheffé test. A p value of < 0.05 was considered statistically significant.

Results

Serum HBD-1 and -2 concentrations. Serum HBD-1 concentrations were higher in patients with lung cancer (11.4±4.2 ng/ml) than in those with pneumonia (6.7±1.8 ng/ml) and normal controls (5.7±1.4 ng/ml), but there was no difference in serum HBD-1 concentrations between patients with pneumonia and normal controls (Figure 1A). The serum concentrations of HBD-2 in patients with lung cancer (378±418 pg/ml) and those with pneumonia (235±253 pg/ml) were higher than in control subjects (36.1±17.0 pg/ml) (Figure 1B). There was no significant relationship between serum levels of HBD-1 and HBD-2 (data not shown).

Comparison of serum HBDs levels before and after surgical resection in patients with lung cancer. Three patients with lung cancer underwent curative surgical resection for lung cancer. Before surgery, the concentration of serum HBD-1, but not HBD-2, was higher in all 3 patients with lung cancer than the mean level of the healthy control. Serum HBD-1 levels after curative resection decreased to levels close to those of the healthy volunteers (Figure 2A). In comparison, the preoperative serum level of HBD-2 was high in one patient with lung cancer and markedly decreased after surgical resection, while the other 2 patients showed only a mild decrease in serum HBD-2 after surgery (Figure 2B).

Immunostaining for HBDs. Immunohistochemical studies showed positive staining for HBD-1 and HBD-2 in bronchial and alveolar epithelial cells in normal lung tissues. HBD-1 staining was also detected in all tumour sections obtained from all 3 patients tested. Likewise, HBD-2 was also detected in tumour cells of all 3 patients (Figures 3 and 4).

Prevalence of tumour markers and correlation with HBDs in lung cancer. To assess whether HBDs are useful as tumour markers, we set the cut-off value of HBD-1 and -2 based on the mean±SD of concentrations determined in our healthy subjects (8.4 ng/ml and 70.1 pg/ml, respectively). Tables I and II list the prevalence of each of the three tumour markers used in the present study and HBDs in lung cancer, according to histopathological classification of the tumour type and

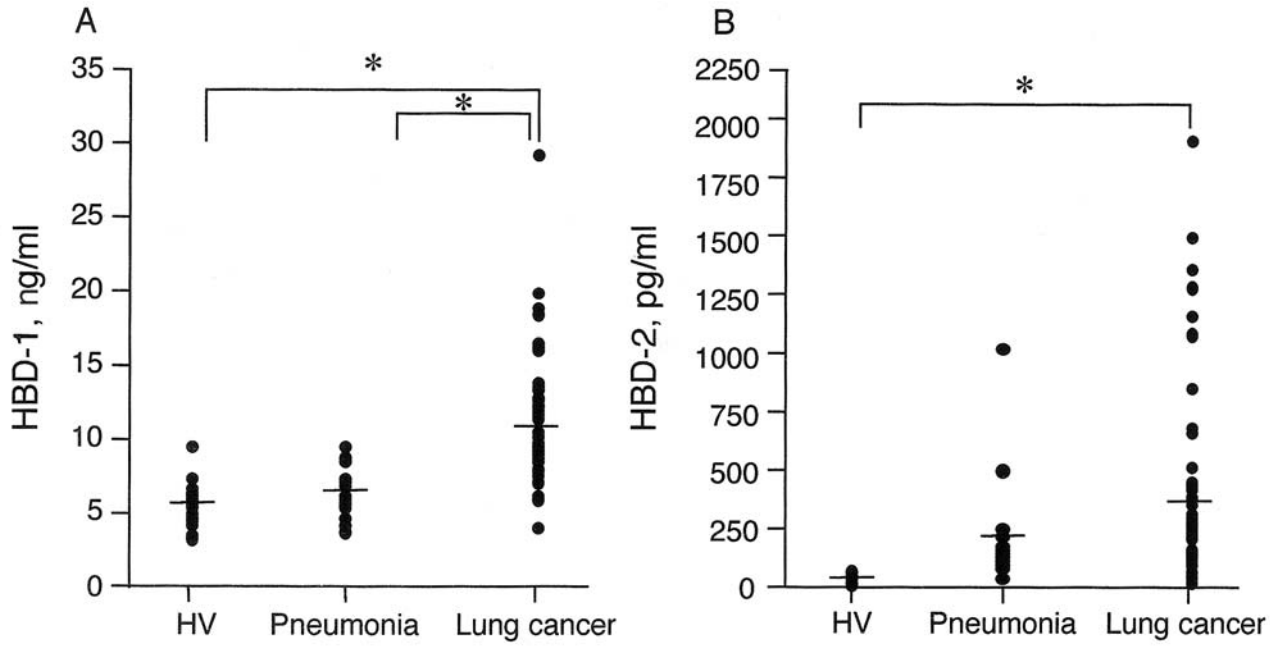

Figure 1. *Serum β-defensins concentrations (A: HBD-1, B: HBD-2) in control subjects, patients with lung cancer and those with pneumonia. Bars represent mean value.*

clinical stage, respectively. The percentages of subjects with serum HBD-1 levels > 8.4 ng/ml were 6, 25, and 76 %, for healthy subjects, patients with pneumonia and patients with lung cancer, respectively. The proportions of patients positive for HBD-1 with clinical stage I and IV were higher than those for CEA, SCC and NSE. On the other hand, the percentages of subjects with serum HBD-2 levels > 70.1 pg/ml were 5, 100 and 86%, for healthy subjects, patients with pneumonia and patients with lung cancer, respectively. The proportions of patients positive for HBD-2 with clinical stage I, IIIA, IIIB and IV, but not stage II, were higher than those for CEA, SCC and NSE. Analysis of prevalence of HBD-1 according to histopathological classification showed a high rate in Ad and Sm tumours. Furthermore, the proportions of patients positive for HBD-2 were 80, 93 and 89% in Ad, Sq and Sm, respectively, which were also higher than CEA, SCC and NSE (Table I). There were no differences in the proportions of patients positive for HBD-1 and HBD-2 based on histopathological classification. The sensitivity and specificity of HBD-1 in patients with lung cancer were 76.4 and 94.0 %, respectively. The sensitivity and specificity of HBD-2 in patients with lung cancer were 85.5 and 95.0%, respectively.

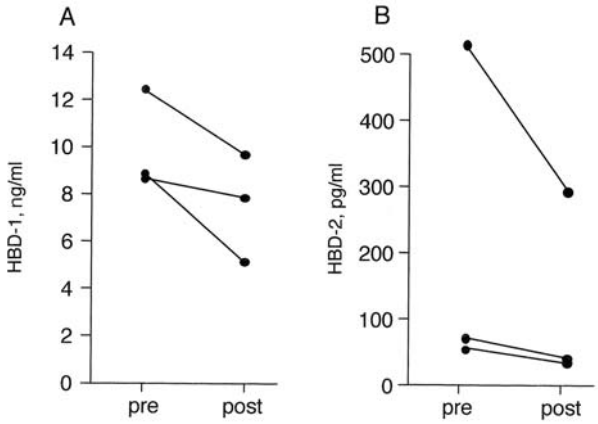

Figure 2. *Serum β-defensins (A: HBD-1, B: HBD-2) measured preoperatively (pre) and postoperatively (post).*

Discussion

The major finding of the present study was that HBDs were expressed by lung cancer cells, resulting in high serum levels of antimicrobial peptides even in the absence of any infection in patients with lung cancer. While we did not confirm the biosynthesis of HBDs in tumour cells, immunohistochemical findings and normalization of high serum HBD-1 levels after curative operation supported the presence of HBD-1 in cancer cells. Lung cancer cells produce several cytokines, a proportion of which play an important role in tumour growth through angiogenesis and stimulation of epidermal growth factor receptor (4,5). Defensins have been recently shown to induce cytokines and act as cytokines. Airway epithelial cells stimulated by defensins release IL-8, which promotes angiogenesis of tumour tissue (15). In our study, there was no correlation between HBD-1 and -2 levels, and HBDs levels in early-stage lung cancer were higher than those in

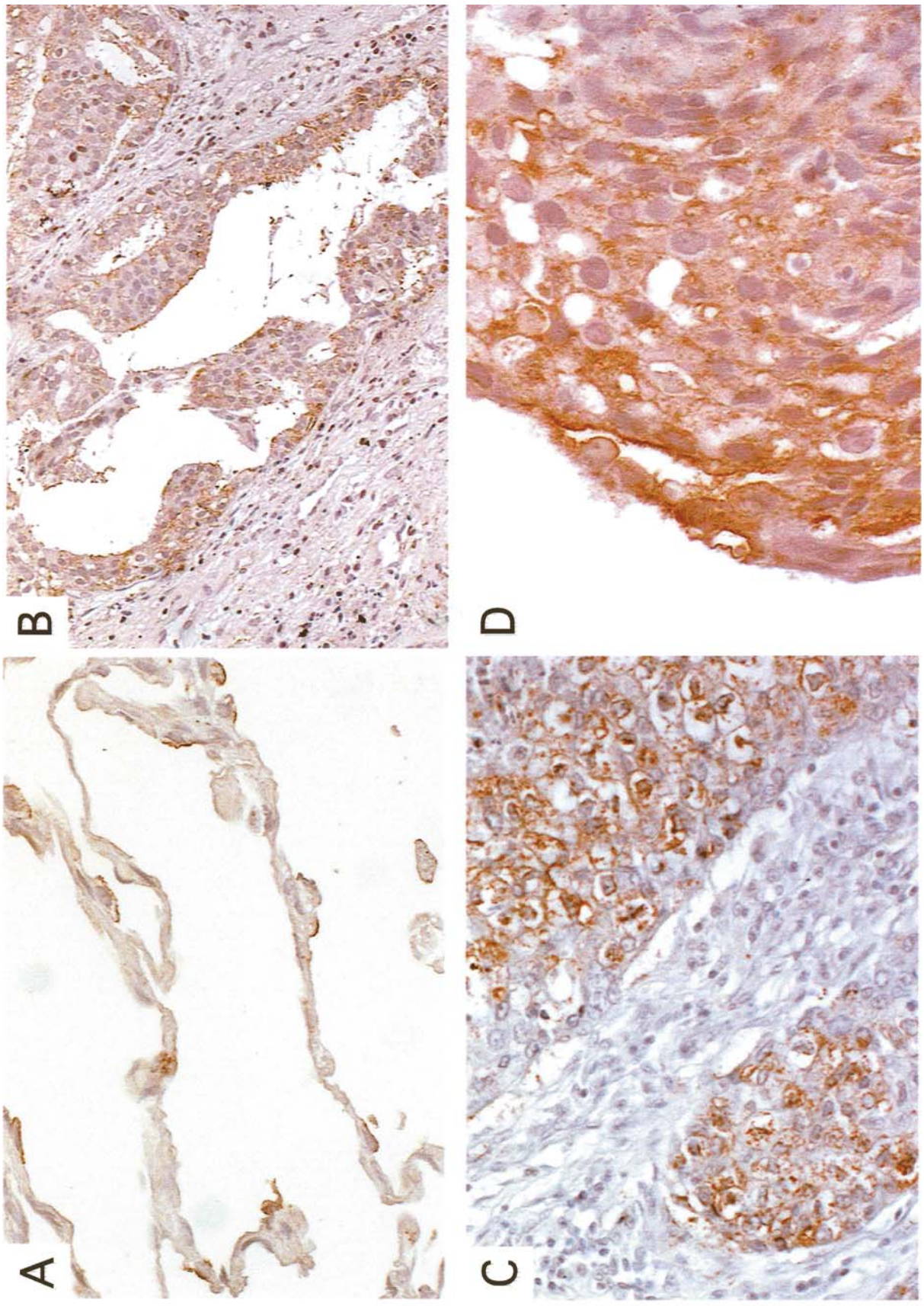

Figure 3. *Immunohistochemical staining of β-defensin 1. Immunostaining was positive in the epithelium of normal lung tissue (A: original magnification x400), tumour cells of adenocarcinoma (B: original magnification x100), squamous cell carcinoma (C: original magnification x100) and large cell carcinoma (D: original magnification x400).*

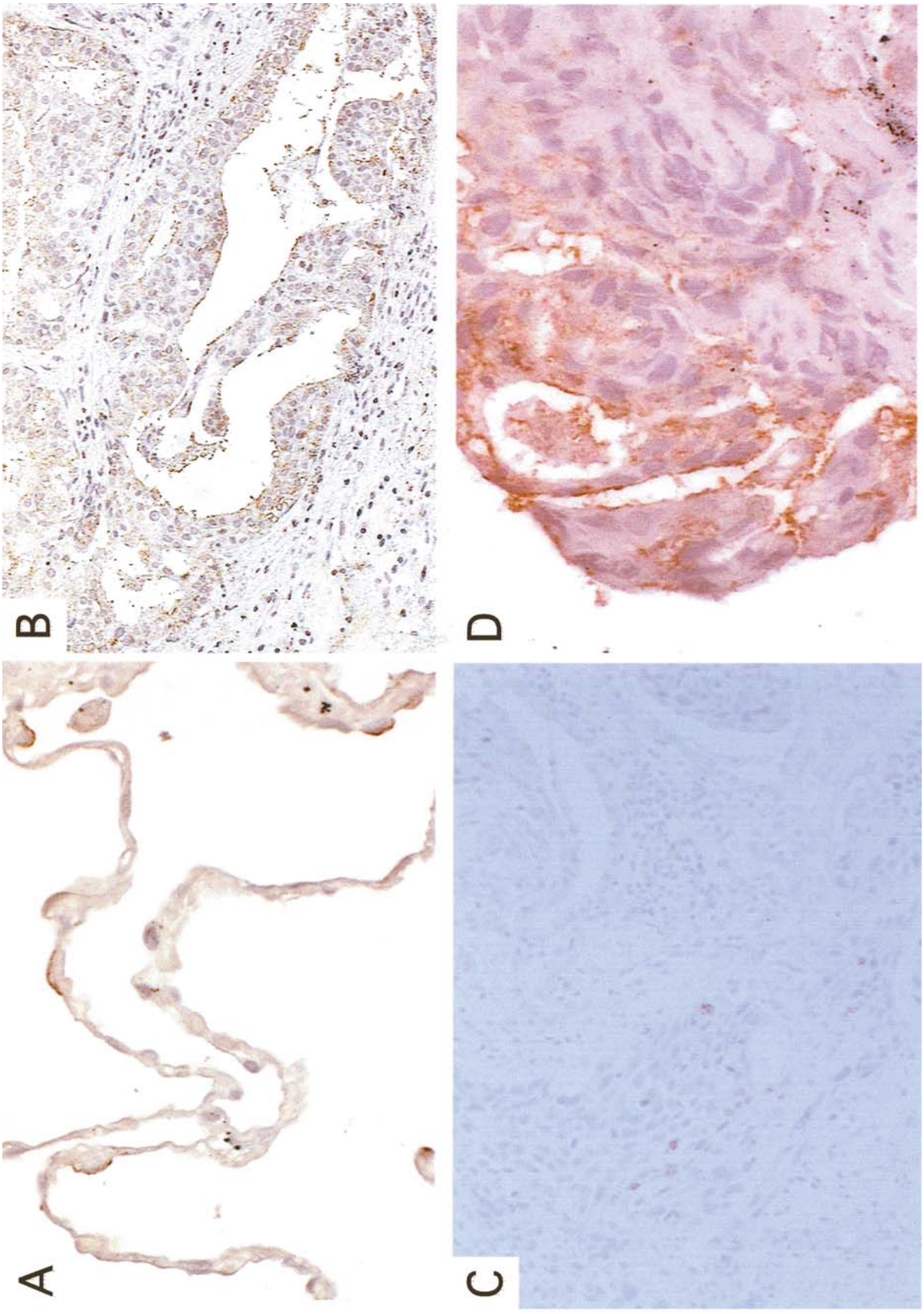

Figure 4. *Immunohistochemical staining of β-defensin 2. Immunostaining was positive in the epithelium of normal lung tissue and tumour cells . For definitions see legend to Figure 3.*

Table I. *Number and proportion of patients positive for various tumour markers and defensins according to histopathological classification of lung cancer*.*

	Whole	Histopathology			
		Ad	Sq	Sm	Lg
CEA (>4.7 ng/ml)**	48.1 (25/52)	51.6 (16/31)	46.2 (6/13)	28.6 (2/7)	100.0 (1/1)
SCC (>1.5 ng/ml)**	39.6 (19/48)	12.0 (3/25)	86.7 (13/15)	28.6 (2/7)	100.0 (1/1)
NSE (>10 ng/ml)**	29.5 (13/44)	20.8 (5/24)	20.0 (2/10)	66.7 (6/9)	0.0 (0/1)
HBD-1 (>8.4 ng/ml)**	76.4 (42/55)	77.4 (24/31)	71.4 (10/4)	77.8 (7/9)	100.0 (1/1)
HBD-2 (>70.1 pg/ml)**	85.5 (47/55)	80.0 (24/30)	93.3 (14/15)	88.9 (8/9)	100.0 (1/1)

*Data are percentage of cases and number of patients positive for the protein/total number of patients tested.
** Level in parenthesis represents the cut-off value for a positive test.

Table II. *Number and proportion of patients positive for various tumour markers and defensins according to the clinical stage of lung cancer*.*

	Whole	Clinical stage				
		I	II	IIIA	IIIB	IV
CEA (>4.7 ng/ml)**	48.1 (25/52)	23.1 (3/13)	50.0 (1/2)	25.0 (1/4)	73.3 (11/15)	50.0 (9/18)
SCC (>1.5 ng/ml)**	39.6 (19/48)	15.4 (2/13)	0.0 (0/2)	25.0 (1/4)	57.1 (8/14)	53.3 (8/15)
NSE (>10 ng/ml)**	29.5 (13/44)	8.3 (1/12)	0.0 (0/2)	25.0 (1/4)	28.6 (4/14)	58.3 (7/12)
HBD-1 (>8.4 ng/ml)**	76.4 (42/55)	69.2 (9/13)	100 (2/2)	50.0 (2/4)	66.7 (10/15)	90.5 (19/21)
HBD-2 (>70.1 pg/ml)**	85.5 (47/55)	84.6 (11/13)	0.0 (0/1)	100 (4/4)	81.3 (13/16)	90.5 (19/21)

*Data are percentage of cases and number of patients positive for the protein/total number of patients tested.
** Level in parenthesis represents the cut-off value for a positive test.

advanced-stage cancer. Therefore, it seemed surprisingly contradictory to the logic that serum HBDs levels reflected only the volume of cancerous tissue. Four types of HBDs are known to be induced by various cytokines. Defensins can cause injury to tumour cells, although the role of HBDs in tumour growth has not been fully elucidated. Further studies are needed to clarify whether the expression of each HBD is linked to a different factor to establish specific biological characteristics such as angiogenesis and tumour growth.

Tumour markers are useful to evaluate the effectiveness of treatment for lung cancer. Our results showed that CEA, NSE and SCC were not suitable markers for early-stage lung cancer as reflected by low positive rates. Especially in stage I lung cancer, previous studies reported that the proportions of patients positive for CEA and SCC were 23% and 22% (3,18). Furthermore, the positive rate of NSE was 33% in the group of limited disease of small cell carcinoma (3). In the present study, serum HBD-2 levels were also high in patients with pulmonary infection as well as those with lung cancer. Therefore, the false-positive rate based on HBD-2 may be higher than that of other tumour markers. However, because serum HBD-1 levels in pneumonia were similar to those of the control subjects and a high proportion of patients with early-stage lung cancer were positive for HBD-1, we propose the use of HBD-1 as a marker for lung cancer, to distinguish it from benign diseases in patients with abnormal shadows on chest X-ray. In the present study, we did not investigate the organ specificity of HBDs. Serum HBD-1 levels were not measured in other cancers apart from lung cancer in the present study. HBD-1 mRNA is expressed in the lungs and kidneys, and HBD-2 mRNA is expressed in lungs and skin (7,8,19).

Serum HBDs levels in lung cancer did not correlate with histopathological classification. CEA, NSE and SCC levels in lung cancer tended to be high in a histopathologically-specific manner in Ad, Sm and SCC, respectively. On the other hand, HBDs levels showed a pattern different from that of the above tumour markers: *i.e.*, no histopathological specificity (Table I). Matrix metalloproteinases (MMPs) as well as HBDs are present in cancers and play a key role in tumour invasion through extracellular matrix degradation. Previous studies showed that overexpression of MMPs induced cancer progression and angiogenesis, which increased the metastatic potential of

malignant tumours (20-24). MMPs are widely expressed in non-small cell lung cancer and small cell cancer irrespective of histological classification (21,22). Several studies reported that MMPs regulate defensins in the normal epithelium (25,26), although it is not clear whether defensins expression is linked to MMPs in cancers. It is possible that MMPs and HBDs expression, which did not correlate with the histopathological classification of lung cancer, are involved in promoting tumour progression through cytotoxicity of defensins on normal cells. Thus, HBDs could be used as common markers for screening lung cancer, especially in early-stage lung cancers.

In conclusion, we have demonstrated in the present study the presence of high serum HBDs levels in patients with lung cancer. HBDs will be expected as a new diagnostic tool for early-stage lung cancer in combination with radiological examination. Further studies are necessary to clarify the exact role of HBDs in tumour growth.

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