The Expression of the CD44 Variant Exon 6 Is Associated with Lymph Node Metastasis in Non-Small Cell Lung Cancer

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ABSTRACT

Recently, it was suggested that splice variants of the surface glycoprotein CD44 (CD44v) were associated with tumor metastasis in some cancers. We examined the expression of variant forms of CD44 in 31 non-small-cell lung carcinomas (NSCLCs) and in 8 normal lung tissue samples by reverse transcription-PCR (RT-PCR). CD44v3, CD44v5, CD44v6, and CD44v7 were not expressed or were weakly expressed in normal lung tissue (0 of 8). In contrast, CD44v3, CD44v5, CD44v6, or CD44v7 was expressed in 28 of 31 (90.3%) NSCLCs. Additionally, we examined the expression of CD44v6, which has been shown to be related to metastasis, in 5 normal lungs and 30 NSCLCs by RT-PCR and immunohistochemical analysis to clarify which cells express CD44v6 in NSCLC specimens. Thirty-six of 61 (59%) NSCLCs variably expressed CD44v6 by RT-PCR, and cancer cells were selectively immunostained by anti-CD44v6 antibodies in 23 of 30 (76.7%) NSCLCs. The results of immunohistochemical analysis almost correlated with those of RT-PCR. NSCLCs with lymph node metastasis expressed significantly more v6 exon than did those without lymph node metastasis [23 of 29 (79.3%) versus 13 of 32 (40.6%); P < 0.01]. There was a significant association between the intensity of v6 expression by RT-PCR and the frequency of cases showing lymph node metastasis (Cochran-Armitage’s test, P < 0.002). In conclusion, this study demonstrated that in NSCLC, a number of variant forms of CD44 are frequently expressed, although these variants are infrequently expressed in normal lung tissue, and that the expression of CD44v6 is particularly associated with lymph node metastasis in NSCLC.

INTRODUCTION

The most life-threatening aspects of the oncogenic process are invasion and metastasis. Lymphogenous or blood-borne metastasis is an early event in patients with lung cancer. It is difficult to treat lung cancer because of the ease of metastasis. A search for tools that allow the metastatic potential of tumors to be effectively assessed is very important for future lung cancer therapies.

It is known that CD44 exists in a standard form, CD44s, and in multiple isoforms, which are generated by alternative splicing of at least 10 variant exons (v1–v10) encoding parts of the extracellular domain. Recently, it was shown that a certain variant exon was associated with tumor metastasis. Analyses of several human carcinomas including colon cancer (1), breast cancer (2), uterine cervical cancer (3), non-Hodgkin’s lymphoma (4), and gastric cancer (5, 6) revealed that the expression of CD44v6 in tumors is related to tumor metastasis and aggressiveness.

Herrlich and coworkers reported that the expression of one particular exon (v6) was correlated with metastatic potential and that transfection of several nonmetastatic tumor cell lines from rats with v6-bearing forms of CD44 caused conversion to a metastatic phenotype (7–9). In human colon cancer, the expression of CD44v6 in tumors was related to lymph node metastasis (10).

Washimi et al. (11) found no correlation between the expression patterns of the various forms of CD44 and the clinicopathological data such as histological type, nodal involvement, and disease stage in NSCLC (11). Givechian et al. (12) also reported that there was no correlation between metastasis and the expression of CD44v forms detected by IHC in bronchial cancers.

We demonstrated that CD44s and CD44v molecules were frequently expressed in NSCLC but were infrequently expressed in small cell lung cancer. In this study, we examined the expression pattern of CD44s and several variant forms of CD44 (CD44v3, CD44v5, CD44v6, CD44v7, and CD44v10) in 31 NSCLCs and 13 corresponding normal lung tissue samples by RT-PCR. We examined the expression of the v6 exon in 61 NSCLCs to clarify the relationship between the expression of the v6 exon, which was shown to be related to metastasis by Herrlich and coworkers, and lymph node metastasis. Moreover, to clarify the relationship between the expression of CD44 molecules by RT-PCR and immunoreactivity by anti-CD44 antibodies, we immunostained 30 NSCLCs and 5 normal lungs by anti-CD44s and v6 antibodies.

MATERIALS AND METHODS

Clinical Specimens. Sixty-one NSCLC samples and 13 corresponding normal lung tissue samples were obtained from...
61 lung cancer patients, who had not received any preoperative adjuvant therapy, during surgery at The Second Department of Surgery, School of Medicine, The University of Tokushima between March 1986 and November 1990. The characteristics of these patients are listed in Table 1. We used the Union International Contre Cancer tumor-node-metastasis staging system to stage the disease (13). Tumor histology was determined according to the WHO classification of lung tumors (14). All patients are listed in Table 1 between March 1986 and November 1990. The characteristics adjuvant therapy, during surgery at The Second Department of Surgery, School of Medicine, The University of Tokushima.

### Table 1: Characteristics of the 61 NSCLC cases used to evaluate CD44 expression

<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>Average</th>
<th>64.1 ± 6.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>47 cases</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>14 cases</td>
</tr>
<tr>
<td>Histology</td>
<td>Sq</td>
<td>25 cases</td>
</tr>
<tr>
<td></td>
<td>Ad</td>
<td>29 cases</td>
</tr>
<tr>
<td></td>
<td>La</td>
<td>7 cases</td>
</tr>
<tr>
<td>Clinical stage</td>
<td>I</td>
<td>24 cases</td>
</tr>
<tr>
<td>T factor</td>
<td>T1</td>
<td>13 cases</td>
</tr>
<tr>
<td>N factor</td>
<td>N1</td>
<td>32 cases</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>10 cases</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>3 cases</td>
</tr>
</tbody>
</table>

Sq, squamous cell carcinoma; Ad, adenocarcinoma; La, large cell carcinoma.

Evaluation of CD44s and CD44v by RT-PCR. After confirmation of the cancerous lesions by H&E staining of a section from frozen blocks, 20 sections (10 μm) were cut from each lesion and collected. Total RNA was extracted from the frozen samples by a single-step procedure using an acid guanidinium thiocyanate-phenol chloroform mixture (15). Single-stranded cDNA was synthesized with a CD44 exon-specific oligonucleotide primer referring to the published CD44 nucleotide sequence (16, 17). This was used for PCR amplification and was homologous to positions 458–483 and 813–843 of the human standard CD44 cDNA (Fig. 1; Ref. 16). These primers anneal to an exon 3 region and an exon 15 region of the CD44 gene (Fig. 1). One of 20 μl of single-stranded template cDNA was used. PCR was carried out for 30 cycles at 94°C (denaturation) for 30 s, 60°C (annealing) for 30 s, and 74°C (elongation) for 1 min. RT-PCR of β-actin was used as the control for each sample. The β-actin-specific oligonucleotide primers used were 5’-ATCACGATGCGATGTCACG-3’ (for RT) and 5’-GTCAGAGAATCTCATTG-3’ and 5’-GCCTGGATGCACGACAT-3’ (for PCR). PCR products from the corresponding tumors and normal lungs were visualized by ethidium bromide (0.5 μg/ml) staining under UV illumination after electrophoresis in a 1.5% agarose gel (Seakem; FMC Bioproducts) in 0.5% Tris-borate EDTA buffer, and then the products were transferred to nylon membranes (Biodyne; Pal Bio Support, East Hills, NY). After prehybridization, the membranes were hybridized overnight at 42°C with a 32P-labeled CD44-S probe (Fig. 1; Refs. 16 and 17). The CD44-S probe was a synthetic oligonucleotide capable of annealing to exon 4, which is on the 5’ side of the alternative splicing site. The membranes hybridized with the CD44-S probe were washed in 2× SSC and 0.1% SDS three times for 5 min at room temperature and then washed in 0.1× SSC and 0.1% SDS twice for 15 min each time at 50°C and subjected to autoradiography. After washing out the CD44-S probe, the same membranes were rehybridized with probes CD44-v3, -v5, -v6, -v7, and -v10 to detect each variant form by the same method (Fig. 1; Refs. 17 and 18).

**Immunohistochemistry.** Frozen sections (6 μm) were fixed in ice-cold acetone (10 min), air-dried for 2 h, and then washed in a Tris-buffered saline solution (0.05 M Tris and 0.15 M NaCl (pH 7.6)) and preincubated with BSA for 5 min. The sections were removed by tapping the glass slides and then incubated with primary antibodies SFF-2 (murine monoclonal antibodies raised against human standard CD44) or VFF-7 (murine monoclonal antibodies raised against an epitope encoded by exon v6 of human variant CD44; Bender, Vienna, Austria; Refs. 3 and 19) at 4°C for 12–16 h. A 1:100 dilution was considered optimal from the titration experiments, using a panel of control tumors. The sections were incubated with the secondary biotinylated antibody for 15 min [antimouse F(ab')2; DAKO Corp.] to form the streptavidin-biotin peroxidase complex. Visualization of the immunocomplex was performed by the immunoperoxidase-3,3′-diaminobenzidine method for 2 min (20), and then the reaction was stopped by adding H2O. The cells were counterstained with hematoxylin, mounted with Malinol, and viewed under the microscope. Background staining activity of the secondary antibody conjugate was excluded by staining control slides without primary antibody. In this study, cases with more than 50% positive tumor cells were defined as “diffusely stained,” those with less than 50% positive tumor cells were defined as “focally stained,” and those without positive tumor cells were defined as “not stained.”

**Intensity of CD44 Expression.** Previously, we examined the expression of the CD44s and CD44v exons in nine NSCLC and five SCLC cell lines by RT-PCR. RNA of the Ma-10 cell line (NSCLC), which strongly expressed CD44s (380 bp) and CD44v exons (870 bp), was amplified by several cycles of RT-PCR (15, 20, 25, 30, 35, and 40 cycles). Because the signals of the RT-PCR products grew exponentially between 25 and 35 cycles, we decided that 30 cycles was the most suitable number of cycles for this study (Fig. 2). We quantified the intensity of CD44s and CD44v6 expressions using NIH image analysis (Version 1.56; Wane Rasband; NIH, Bethesda, MD) to clarify the relationship between the frequency of lymph node metastasis and the intensity of v6 expression. Because the average intensity of the CD44s bands was 2529 ± 236 (mean ± standerror of the mean) to clarify the relationship between the frequency of lymph node metastasis and the intensity of v6 expression. Because the average intensity of the CD44s bands was 2529 ± 236 (mean ±
The intensity of CD44s in each sample was used as an internal control. Cases that did not express v6 were classified as the none group. Cases with a numerical value of less than 2000 were classified as the weak group, cases with a numerical value of more than or equal to 5000 were classified as the strong group, and cases with a numerical value of more than or equal to 5000 were classified as the strong group.

**Statistical Analysis.** The relationship between the incidence of CD44s and CD44v exon expression and several clinical factors was analyzed by χ² test, Fisher's exact test, Student's t-test (unpaired), or Welch test. Correlations between the intensity of CD44v6 expression and the frequency of lymph node metastasis were examined by using the Cochran-Armitage's test. Correlations were considered to be statistically significant at P < 0.05.

**RESULTS**

**Expression of the CD44 Isoforms in Primary Lung Cancers and the Corresponding Normal Lung Tissue.** The electrophoretic profiles of the PCR products of the CD44 gene in eight lung cancer specimens and in the corresponding normal lung tissue are shown in Fig. 3A. One PCR product had almost the same intensity as the 382-bp fragment band, and a variety of bands (greater than 382 bp) had variable intensities. Some bands that were less than 382 bp were thought to be nonspecific bands for the CD44 gene. Each sample was amplified with β-actin primers before CD44 amplification to check the quality and abundance of the RNAs. PCR for β-actin showed that the amounts and quality of mRNA did not differ significantly among the 16 samples.

The PCR products obtained from electrophoresis were transferred onto nylon membranes. The membranes were hybridized with the CD44-S probe that detected exon 4. After washing out the CD44-S probe, the same membranes were rehybridized with the probe for CD44-Vs to detect the variant forms (Fig. 3B). All pairs of normal lung and lung cancer specimens were found to express CD44s, which was the 382-bp fragment, and the intensity of the CD44s expression of normal lung tissue was almost the same as that in the lung cancer specimens. But the expression of the variant forms of CD44 differed between normal tissue and the cancer specimens. With the CD44-v5 probe, five of eight cancer cases expressed CD44v5, and the intensity of CD44v5 expression varied. None of the normal lung cancer cases expressed it. With the CD44-v5 probe, none of the eight cancer cases expressed CD44v5. With the CD44-v6 probe, none of the eight normal lung cases expressed CD44v6 but seven of eight cancer cases expressed CD44v6. Although the intensity of expression varied from case to case, CD44v7 expression paralleled CD44v6 expression in the same cases. With the CD44-v10 probe, all of the cancer cases expressed CD44v10. Four of eight (50%) normal lung cases expressed it, but CD44v10 expression in the normal lungs was weaker than that in the corresponding cancers.

**Expression of CD44 Isoforms in Primary Lung Cancers.** We examined the expression of CD44s and the CD44v exons in an additional 23 NSCLCs to determine the alternative splicing pattern of the CD44 gene in lung cancer. All lung cancer cases were found to express CD44s, and the intensity of CD44s was almost the same in all of the lung cancer cases. The frequency of variant exon expression in the 31
NSCLC patients is shown in Fig. 4. With the CD44-v3 probe, 26 of 31 (83.9%) of the cases expressed CD44v3. The frequency of CD44v3 expression in adenocarcinoma was higher than that in squamous cell carcinoma (93.3 versus 66.7%). With the CD44-v5 probe, 12 of 31 (38.7%) of the cases expressed CD44v5. With the CD44-v6 and -v7 probes, 26 of 31 (83.9%) cases expressed CD44v6 and CD44v7. Although the intensity of expression varied from case to case, CD44v6 expression paralleled that of CD44v7 in the same patients. With the CD44-v10 probe, 30 of 31 (96.8%) cases expressed CD44v10. The frequency of CD44v5, CD44v6, CD44v7, and CD44v10 expression in adenocarcinoma was about the same as that in squamous cell carcinoma.

**Relationship between Clinical Findings and the Expression of Variant Exon 6 in NSCLC.** In 31 NSCLCs that were examined for expression of each variant exon by RT-PCR, there was no relationship between v3, v5, v6, v7, or v10 expression and clinico-pathological features including age, gender, Brinkman index, histology, T factor, and clinical stage (data not shown). Only v6 and v7 expressions were associated with lymph node metastasis. The expression of other exons was not associated with lymph node metastasis (Table 2). Because the expression of v6 paralleled that of v7, the expression of CD44v6 was assayed by RT-PCR in an additional 5 normal lung and 30 NSCLC specimens to confirm the association between v6 expression and lymph node metastasis. In total, 2 of 13 (15%) normal lung samples weakly expressed the CD44v6 exon. Conversely, 36 of 61 (59%) NSCLCs expressed CD44v6. Thirteen of 32 (40.6%) NSCLCs without lymph node metastasis expressed v6, as shown by RT-PCR, whereas 23 of 29 (79.3%) NSCLCs with lymph node metastasis expressed v6. A statistically significant association was seen only between the expression of the v6 exon and lymph node metastasis \(P < 0.01\); Table 3). There was no correlation between the expression of CD44v6 and the other clinico-pathological parameters.

**Correlation between Lymph Node Metastasis and the Intensity of CD44v6 Expression.** We quantified the intensity of CD44s and CD44v6 expression by NIH image analysis to clarify the association between the frequency of lymph node metastasis and the intensity of v6 expression. Because the intensity of CD44s expression in all lung cancers was almost the same (Fig. 3A), that is, the average intensity of CD44s was 2529 ± 236 (mean ± SD), the intensity of CD44s was used as an internal control. The 61 NSCLCs examined were divided into four groups, according to the intensity of CD44v6 expression. Six of 25 (25%) cases in the none group had lymph node metastasis. Two of 5 (40%) cases in the weak group, 10 of 15 (67%) cases in the moderate group, and 11 of 16 (69%) cases in the strong group had lymph node metastasis. There was a significant association between the intensity of v6 expression and the frequency of lymph node metastasis (Cochran-Armitage’s test, \(P < 0.002\); Fig. 5).
Immunohistochemical Detection of CD44 in the Normal Lung and in Cancerous Lung Tissue. To clarify the association between CD44s and v6 expression of RT-PCR and that of immunostain, cryostat sections from 5 of 13 normal lungs examined by RT-PCR and 30 of 61 NSCLCs examined by RT-PCR were immunostained with anti-CD44s- and v6-specific antibodies. In the normal lung, all five specimens expressed CD44s by RT-PCR; type I and II pneumocytes, interstitial tissues, and macrophages were stained by anti-CD44s antibodies (Fig. 6A). Three normal lungs that did not express CD44v6 by RT-PCR were not stained by anti-CD44v6 antibodies, but two normal lungs that expressed small amounts of v6 by RT-PCR showed focal staining of type II pneumocytes by anti-CD44v6 antibodies (Fig. 6B). In the NSCLC cases, the tumor cells as
Squamous cell ca. (n=12)

Adenoca. (n=15)

Large cell ca. (n=4)

Non-small cell ca. (n=31)

Fig. 4 The frequency of CD44v exon expression in 31 NSCLC cases.

well as the interstitial tissue were stained by anti-CD44s antibodies, and the intensity of CD44s immunostain was almost the same in all NSCLC cases (Fig. 6, C and E). However, seven cases that did not express CD44v6 by RT-PCR did not show any immunostain with anti-CD44v6 antibodies. Twenty-three cases that expressed CD44v6 by RT-PCR were focally or diffusely immunostained. The membrane of the tumor cells was selectively stained by anti-CD44v6 antibodies. The fibroblast and infiltrating leukocytes in the stroma were not stained. The three cases in the weak group on RT-PCR were focally immunostained. In the moderate and strong groups, 14 cases were focally immunostained, and 6 cases were diffusely stained (Fig. 6, D and F; Table 4). There was a correlation between the immunoreactivity detected by anti-CD44s and v6 antibodies and the expression of CD44s and v6 on RT-PCR.

DISCUSSION

CD44 is an integral membrane glycoprotein that possesses a number of isoforms generated by alternative splicing of 10 variant exons. Several studies investigated the expression of CD44s and variant exons in lung cancer (21, 22). We also analyzed the expression of CD44s and variant exons in lung cancer cell lines and specimens (data not shown). Our results were similar to those of previous studies (21, 22). These results suggested that CD44s and variant exons were frequently expressed in NSCLC but were not expressed in small cell lung cancers and that the metastasis of NSCLC may be related to CD44 molecules. In this study, we focused on the expression of CD44s and CD44v in NSCLC and examined the relationship between CD44v6 expression and lymph node metastasis in NSCLC.

Interestingly, CD44v exons are frequently expressed in lung cancer but not in the normal lungs, whereas CD44s is expressed to the same degree not only in normal lungs but also in cancerous lungs. CD44v exons, except v10, were not expressed or were weakly expressed in normal lung tissue, as shown by RT-PCR, although 2 of 13 normal lungs very weakly expressed CD44v6. In contrast, CD44v3, CD44v5, CD44v6, or CD44v7 was expressed in 28 of 31 (90.3%) NSCLCs. This study demonstrated that in two normal lungs that weakly expressed CD44v6, as shown by RT-PCR, some type II pneumocytes were immunostained by anti-CD44v6 antibodies. Using a different antibody than we did, Kasper et al. (23) reported that the CD44v6 isoform was detectable in type II pneumocytes and in basal cells of the bronchial epithelium. Although some tissues in normal lung express CD44v, as shown by IHC, the RT-PCR conditions in this study detected little or no CD44v expression in normal lungs. However, we detected variable CD44v expression in many of the NSCLCs. Thus, our results suggest that the expression of CD44v on RT-PCR may be a useful marker for the early diagnosis of primary NSCLC or of local recurrence.

NSCLC had a wide variety of CD44v expression patterns. About 80% of NSCLCs expressed variant exons v3, v6, and/or v7 variably. The frequency of CD44v5 expression in NSCLC was less than that of the other variant exons. Conversely, CD44v10, which was one exon of the epithelial form, was expressed in most NSCLCs (30 of 31, 96.8%). It is known that CD44 molecules have various functions that are attributed to the various isoforms. Although no ligand has yet been identified for the region encompassing variant exons v1–v10, a hydrophilicity plot of the CD44 molecule revealed that the hydrophilic area was predominantly concentrated in variant exons v1–v9. Because the hydrophilic regions of a protein usually face outward, these regions are potential candidates for regions interacting with ligands (24). This review leads us to the assumption that NSCLC cells may have some advantages for growth, invasion, and metastasis by gaining the multifunctions for cell-cell and/or cell-matrix interactions induced by a wide variety of alternative splicing patterns.

The frequency of v6 expression in NSCLCs with lymph node metastasis was significantly more than that in NSCLCs without lymph node metastasis [23 of 29 (79.3%) versus 13 of 32 (40.6%); P < 0.01]. Furthermore, there was a correlation between the intensity of CD44v6 expression in NSCLCs and...
Table 2: Correlation between N factor and the expression of CD44s, CD44v3, CD44v5, CD44v6, CD44v7, and CD44v10 by RT-PCR in 31 NSCLCs

<table>
<thead>
<tr>
<th>N factor</th>
<th>CD44s</th>
<th>CD44v3</th>
<th>CD44v5</th>
<th>CD44v6</th>
<th>CD44v7</th>
<th>CD44v10</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>13</td>
<td>0</td>
<td>9</td>
<td>4</td>
<td>5</td>
<td>8</td>
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<tr>
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<td>3</td>
</tr>
<tr>
<td>N2</td>
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<td>0</td>
<td>10</td>
<td>0</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>N3</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

* NS, not significant. Fisher’s exact test.
+ expression by RT-PCR.
- no expression by RT-PCR.
P<0.01° Fisher’s exact test.

Table 3: Correlation between several factors and the expression of the CD44v6 in NSCLCs

<table>
<thead>
<tr>
<th>CD44v6</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs: average)</td>
<td>65.5 ± 8.0</td>
<td>62.1 ± 9.0</td>
</tr>
<tr>
<td>Gender</td>
<td>Male 28 (59.6%)</td>
<td>19 NS*</td>
</tr>
<tr>
<td></td>
<td>Female 8 (57.1%)</td>
<td>6 NS*</td>
</tr>
<tr>
<td>BI (average)</td>
<td>835 ± 696</td>
<td>832 ± 416</td>
</tr>
<tr>
<td>Stage</td>
<td>I 11 (45.5%)</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>II 4 (100%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IIIA 12 (60%)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>IIIB 5 (62.5%)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>IV 4 (80%)</td>
<td>1</td>
</tr>
<tr>
<td>T factor</td>
<td>T1 7 (54%)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>T2 24 (67%)</td>
<td>12 NS*</td>
</tr>
<tr>
<td></td>
<td>T3 2 (33%)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>T4 3 (50%)</td>
<td>3</td>
</tr>
<tr>
<td>N factor</td>
<td>N0 13 (40.6%)</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>N1 7 (100%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>N2 13 (68.4%)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>N3 3 (100%)</td>
<td>0</td>
</tr>
</tbody>
</table>

* NS, not significant; t-test.
† x² test.
‡ Welch test.

For instance, if a case is preoperatively diagnosed as NSCLC and shows strong expression of CD44v6 on a biopsy specimen, that case should undergo adequate dissection of the mediastinal and hilar lymph nodes.

In lung cancer, Washimi et al. (11) reported no correlation between the expression patterns of CD44v forms and nodal involvement in NSCLC. The results of Washimi et al. (11) do not concur with our results. They used a probe corresponding to CD44v4--v7 and demonstrated that not only NSCLC but also normal lung tissue strongly expressed either CD44v4, CD44v5, CD44v6, or CD44v7. In their study, the contamination with normal cells may have caused false positive results. In contrast, we demonstrated the specific expression of CD44v6 in NSCLC.

In our study, CD44v6 expression in normal lung tissue was either nonexistent or very weak, as shown by RT-PCR. Only tumor cells in NSCLC expressed CD44v6, but the fibroblasts and infiltrating leukocytes in the stroma of NSCLC did not express CD44v6.
express CD44v6 on IHC, as shown in Fig. 6, D and F. If there was contamination with normal cells, our assay did not detect the false positive. Therefore, we think that the results of Washimi et al. (11) did not pinpoint CD44v6 expression in NSCLC cancer cells and that their findings were influenced by exons other than v6 and by normal components in lung cancers.

Several studies have demonstrated that the expression of the v6 exon is correlated with the lymphatic metastatic potential. In colorectal tumors, an epitope encoded by CD44v6 correlates with tumor progression, being the highest in metastasizing tumors (Dukes’ stages C and D) and has a poor prognosis (10). In breast cancers with lymph node metastasis, expression of sev-
The expression of the CD44v6 by RT-PCR and IHC in NSCLCs

<table>
<thead>
<tr>
<th>IHC$^{b}$</th>
<th>None</th>
<th>Weak</th>
<th>Moderate</th>
<th>Strong</th>
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<tr>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>++</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

$^{a}$ Intensity quantified by NIH image as: none, no expression; weak, <2000; moderate, 2000–5000; strong, >5000.

$^{b}$ - , no stain; +, focally stained (positive cells are less than 50% of cancer cells); ++, diffusely stained (positive cells are more than 50% of cancer cells).

eral splice variants was strong, but in tumors without lymph node metastasis, signal intensity was weaker (2).

In conclusion, this study demonstrated that in NSCLC, a variety of CD44 exons are frequently expressed, but the normal lung infrequently expresses them, and that the expression of CD44v6 is associated with lymph node metastasis. The functions of the CD44 exons, including v6, need to be examined further to elucidate the adhesive features of NSCLC. The discovery of the CD44v6 ligand may also provide a strategy for overcoming the metastasis of NSCLC cells in the lymph nodes.

**ACKNOWLEDGMENTS**

We thank Prof. T. Sano (First Department of Pathology, School of Medicine, The University of Tokushima) for his pathological analysis and helpful discussion.

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