Ann Surg Oncol (2019) 26:1544–1551
https://doi.org/10.1245/s10434-018-07137-2

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

The Significance of CD44 Variant 9 in Resected Lung Adenocarcinoma: Correlation with Pathological Early-Stage and *EGFR* Mutation

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ABSTRACT

Background. CD44 isoforms serve as a marker for cancer stem cells. CD44 variant 9 (CD44v9) contributes to the defense against reactive oxygen species, resulting in resistance to chemoradiotherapy. However, the significance of CD44v9 in patients with lung adenocarcinoma is unknown.

Methods. We used immunohistochemical analysis to retrospectively analyze CD44v9 expression in 268 surgically resected lung adenocarcinomas and investigated the association between CD44v9 expression and patients' clinicopathological features.

Results. The expression of CD44v9 in 193 of 268 (72.0%) patients was significantly associated with early-stage cancer, low-grade tumors, absence of vessel and pleural invasion, and a mutated epidermal growth factor receptor (*EGFR*) gene. Multivariate logistic analysis revealed that CD44v9 expression was significantly associated with early-stage disease [odds ratio (OR) 0.29, 95% confidence interval (CI) 0.14–0.59; $p < 0.001$] and mutant *EGFR* (OR 2.53, 95% CI 1.06–6.04; $p = 0.036$). The percentage of CD44v9-positive tumors was higher in the earlier stages of disease; however, there was no significant difference in the survival of patients in each stage of disease who had positive or negative CD44v9 expression.

Conclusion. CD44v9 was highly expressed in *EGFR*-mutant tumors, particularly in early-stage lung adenocarcinoma, suggesting that CD44v9 expression may play an important role in *EGFR*-mutant tumors.

The therapeutic options for lung cancer, particularly non-small cell lung cancer (NSCLC), have expanded during the past decade. The identification of driver oncogenes, such as a mutated epidermal growth factor receptor (*EGFR*) gene, or rearrangement of the anaplastic lymphoma kinase gene, has prolonged the survival of patients through the development of specific tyrosine kinase inhibitors (TKIs) that are now first-line therapies for patients with advanced NSCLC who harbor these genetic alterations.[1] Immune checkpoint inhibitors targeting programmed death 1/programmed death ligand 1 or cytotoxic T-lymphocyte antigen 4 now serve as standard treatments of lung cancer.[2,3] However, the prognosis of patients with NSCLC is poor, despite the administration of such therapies.[3] The failure of treatment and the relapse of cancer may be explained by drug resistance and the self-renewal of cancer stem cells (CSCs).[4,5]

The cell adhesion molecule CD44, which serves as a marker of CSCs, is a transmembrane hyaluronan-binding glycoprotein that is involved in multiple cellular functions, such as the regulation of cell proliferation, adhesion, invasion, metastasis, and drug resistance.[6,7] *CD44*

Electronic supplementary material The online version of this article (https://doi.org/10.1245/s10434-018-07137-2) contains supplementary material, which is available to authorized users.

First Received: 26 September 2018;
Published Online: 24 February 2019

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comprises 20 exons, of which exons 6–15 are referred to as variant exons v1–v10.[6] Alternative splicing inserts the variant exons, in various combinations, into the *CD44* transcript, which is controlled by the epithelial splicing regulatory proteins (ESRP) 1 and 2, to generate CD44 isoforms (CD44v).[8]

Although the functions of each variant isoform are unknown, the expression of CD44 variants containing exons 8–10 (CD44v8–10 or CD44v9) contributes to the defense against reactive oxygen species (ROS).[9,10] These CD44v isoforms stabilize the expression of xCT, a subunit of a cystine-glutamate antiporter, which upregulates the synthesis of the intracellular antioxidant glutathione from imported cystine, thus contributing to the resistance of tumor cells to chemotherapy and radiotherapy.[9,10] Although the significance of CD44v9 expression was investigated in breast, colon, head and neck, ovarian, bladder, and gastric cancers[11–14] we are unaware of studies of its expression in lung cancer.

The contribution of CD44v to chemoresistance led to phase I clinical studies targeting CD44v-positive cancer cells in patients with advanced gastric cancer.[15] Recently, a phase I study of patients with advanced NSCLC evaluated salazosulfapyridine, a specific inhibitor of xCT-mediated cystine transport, combined with cytotoxic chemotherapy;[16] however, the biological significance of CD44v in NSCLC is unknown.

To address this gap in our knowledge, we measured the expression of CD44v9 in surgically resected primary lung adenocarcinomas and investigated the associations of CD44v9 expression with patients' clinicopathological features.

MATERIALS AND METHODS

Patients and Samples

We retrospectively examined patients with primary lung adenocarcinoma who underwent surgical resection between January 2003 and December 2013 at the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University. We retrieved 268 paraffin-embedded specimens from the registry of the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University. Patients' clinicopathological features included age on surgery, sex, smoking habits, pathological tumor-node-metastasis (TNM) stage (7th edition of the Lung Cancer Staging System), histological tumor grade, and pleural or lymphovascular invasion. Histological tumor grades were categorized as follows: G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated. The presence of *EGFR* mutations in 197 samples of tumor tissue specimens was determined using the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp method (Mitsubishi Chemical Medicine, Tokyo, Japan).[17] The clinical information and follow-up data were obtained from patients' medical records. This study was approved by the Institutional Review Board (IRB) at Kyushu University (IRB No. 30-55).

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was performed using 4-μm-thick sections of paraffin-embedded tissue. We selected paraffin-embedded blocks containing the most tumor cells in the primary tumors. The sections were deparaffinized in xylene and dehydrated in an ethanol series. For antigen retrieval, the specimens were pretreated in Target Retrieval Solution (S-1699; Dako Japan, Tokyo, Japan) in an autoclave at 105 °C for 10 min for CD44v9 staining, and at 121 °C for 5 min for panCD44 staining. The sections were incubated for 30 min in 0.3% hydrogen peroxidase in absolute methanol to deactivate endogenous peroxidases. After nonspecific antibody binding was inhibited by incubating the sections in 10% normal rabbit serum, the specimens were incubated with a rat anti-CD44v9 monoclonal antibody (clone RV3, 1:5000 dilution; Cosmo Bio, Tokyo, Japan) and rabbit anti-panCD44 monoclonal antibody (clone EPR1013Y, 1:50 dilution; Abcam, Cambridge, UK) at 4 °C overnight. For CD44v9 staining, the sections were washed and incubated for 30 min in biotinylated rabbit anti-rat IgG (1:400 dilution; Abcam). Finally, bound primary antibodies were detected using a DAKO EnVision Detection System-HRP Labelled Polymer Anti-Rabbit (K4003; Dako Japan, Tokyo, Japan). The sections were then reacted with 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted.

Evaluation of IHC Data

The levels of CD44v and panCD44 expression were evaluated by two investigators (TA and KI) using the Allred scoring system.[18] Diffuse and focal staining of tumor cells was observed in the basal layer of the epithelium and cellular membrane, but not in intracellular compartments (Fig. 1a and electronic supplementary Figure 1A). Therefore, carcinoma cells that expressed CD44v9 and panCD44 throughout the entire circumference of the membrane were defined as positive. Figure 1a shows representative examples of negative CD44v9 expression, while Fig. 1b shows positive expression. Representative examples of panCD44-negative and -positive expression are shown in electronic supplementary Figure 1A and 1B,

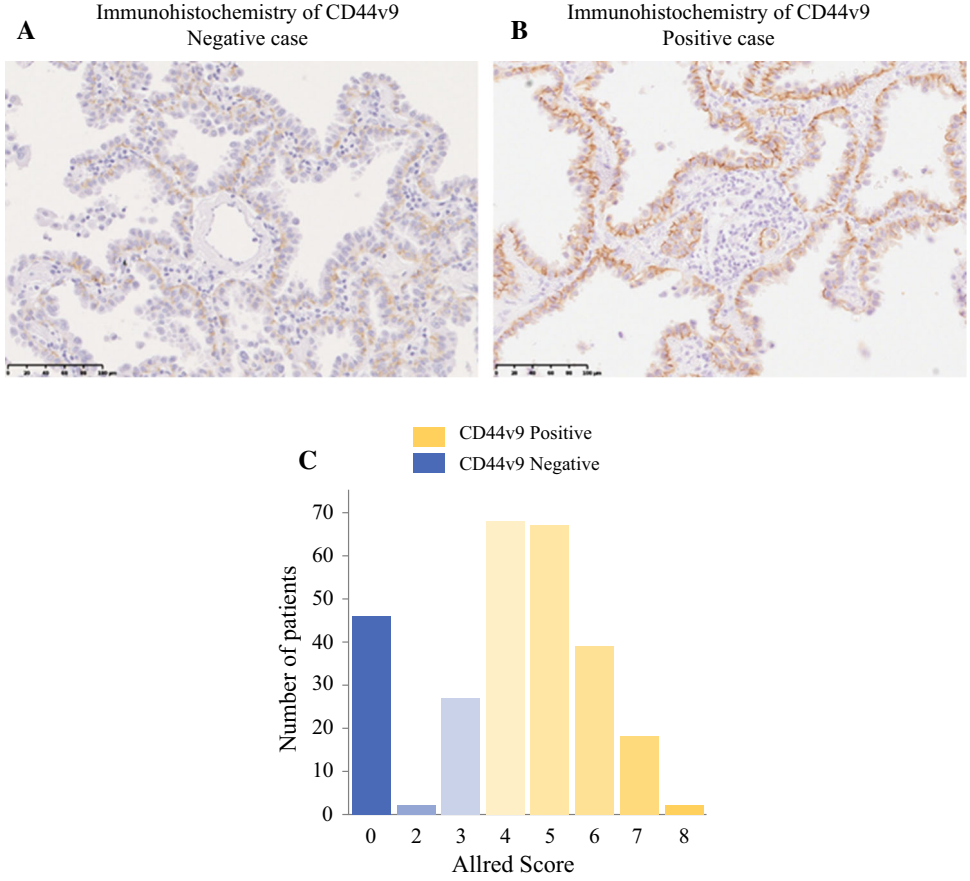

FIG. 1 Immunohistochemical analysis of CD44v9 expression in tumor cells of patients with lung adenocarcinoma. **a** Negative example of CD44v9 expression showing diffuse and focal staining in the basal layer of the epithelium. **b** Positive example of CD44v9 expression showing staining of the entire circumference of the cell membrane. **c** Histogram of CD44v9 Allred scores of all patients with lung adenocarcinoma

respectively. Briefly, the total Allred score determined for intensity (absent, 0; weak, 1; moderate, 2; and strong, 3) was added to the score assigned to the percentage of tumor cells for CD44v9 and panCD44 staining (no cells, 0; < 1% of cells, 1; 1–10% of cells, 2; 11–33% of cells, 3; 34–66% of cells, 4; and 67–100% of cells, 5). The histograms of the CD44v9 Allred scores showed a bimodal distribution, therefore total Allred scores of 0–3 were defined as negative expression, and scores of 4–8 were defined as positive expression (Fig. 1c). A cut-off score of 4 was also used for panCD44 expression.

Statistical Analysis

Statistical analyses of categorical factors were performed using Fisher's exact test, and univariate and multivariate analyses of the relationships between CD44v9 expression and other patient characteristics were performed using logistic regression analysis. Disease-free survival (DFS) was defined as the period between surgery and the date of the recurrence or progression, which was determined according to the Response Evaluation Criteria in Solid Tumors or death from any cause. Overall survival (OS) was defined as the period between surgery and the date of the last follow-up or death. These rates were estimated using the Kaplan–Meier method with the log-rank test. All statistical analyses were performed using JMP Statistical Discovery Software version 11.0 (SAS Institute, Cary, NC, USA), and a p value < 0.05 was considered statistically significant.

RESULTS

Association Between CD44v9 Expression and Clinicopathological Characteristics of Patients with Primary Lung Adenocarcinoma

Electronic supplementary Table 1 presents the patients' characteristics. Overall, 139 (51.9%) patients were men, 135 (50.3%) were never smokers, the median age was 68 years (range 29–87), 101 (51.2%) had wild-type *EGFR*, and 96 (48.7%) had a mutant *EGFR*. The associations between CD44v9 expression and clinicopathological characteristics of patients are presented in Table 1. CD44v9 expression, which was detected in 193 (72.0%) samples, was significantly associated with early-stage cancers (including pathological T and N factors), low-grade tumors, and the absence of vessel and pleural

TABLE 1 Association between CD44v9 expression and clinicopathological factors of patients with lung adenocarcinoma

Factors		*N*	CD44v9 [*N* (%)]		*p* value
			Negative	Positive	
Age (years)	< 70	151	44 (58.7)	107 (55.4)	0.682
	≥ 70	117	31 (41.3)	86 (44.6)	
Sex	Female	139	37 (49.3)	102 (51.5)	0.683
	Male	129	38 (50.7)	91 (47.2)	
Smoking history	Never-smoker	135	38 (50.7)	97 (50.3)	1
	Smoker	133	37 (49.3)	96 (49.7)	
Pathological T status	T1	132	24 (32.0)	108 (55.9)	< 0.001
	≥ T2	136	51 (68.0)	85 (44.1)	
Pathological N status	N0	183	29 (38.7)	154 (79.8)	< 0.001
	≥ N1	85	46 (61.3)	39 (20.2)	
Pathological stage	I	158	21 (28.0)	137 (71.0)	< 0.001
	≥ II	110	54 (72.0)	56 (29.0)	
Grade	G1	119	20 (26.7)	99 (51.3)	< 0.001
	G2, G3	149	55 (73.3)	94 (48.7)	
Pleural invasion	Absent	195	45 (60.0)	150 (77.7)	0.006
	Present	73	30 (40.0)	43 (22.3)	
Lymphatic invasion	Absent	214	47 (62.7)	167 (86.5)	< 0.001
	Present	54	28 (37.3)	26 (13.5)	
Vascular invasion	Absent	175	37 (49.3)	138 (71.5)	< 0.001
	Present	93	38 (50.7)	55 (28.5)	
EGFR[a]	Wild-type	101	28 (66.7)	73 (47.1)	0.036
	Mutant	96	14 (33.3)	82 (52.9)	

[a]Patients with data

EGFR epidermal growth factor receptor

invasion. A mutant *EGFR* was also significantly associated with high CD44v9 expression ($p = 0.036$).

Univariate and Multivariate Analyses of the Association between CD44v9 Expression and Clinicopathological Factors in a Cohort of Patients Whose EGFR Status Data Were Available

We furthermore examined the association between CD44v9 expression and other patient characteristics in a cohort of 207 patients whose *EGFR* status data were available. In multivariate logistic analysis, CD44v9 expression was significantly associated with early-stage disease [odds ratio (OR) 0.29, 95% confidence interval (CI) 0.14–0.59; $p < 0.001$] (Table 2) and mutant *EGFR* (OR 2.53, 95% CI 1.06–6.04; $p = 0.036$) (Table 2). In addition, CD44v9 expression correlated with various phases of TNM stages (electronic supplementary Figure 2). There was a significantly higher proportion of CD44v9-positive patients in the early stages of disease [stage I, $n = 137$ (86.2%); stage II, $n = 35$ (60.4%); stage III–IV, $n = 21$ (41.2%); $p < 0.001$].

Association of Survival of Patients Who Underwent Surgical Resection of Primary Lung Adenocarcinoma with Stage-Specific CD44v9 Expression

The association between pathological stages and CD44v9 expression led us to search for a stage-specific association of CD44v9 expression with survival. There were no significant differences associated with pathological stage in OS and DFS between CD44v9-positive and CD44v9-negative patients (electronic supplementary Figure 3). Although the sample size was too small to analyze, the median value of DFS was slightly better in patients without than in those with CD44v9 expression in stage II (4.14 vs. 2.16 years) (electronic supplementary Figure 3C) and stage III–IV (1.42 vs. 1.10 years) (Fig. 2a).

TABLE 2 Univariate and multivariate analyses of CD44v9 expression and clinicopathological factors in a cohort of patients whose *EGFR* status data were available

Factors		Total (*N*)	CD44v9-positive [*N* (%)]	Univariate analysis [OR (95% CI)] *p* value	Multivariate analysis [OR (95% CI)] *p* value
Age (years)	≥ 70	87	69 (79.3)	1.07 (0.54–2.13)	1.22 (0.57–2.57)
	< 70	110	86 (78.2)	0.848	0.610
Sex	Male	91	70 (76.9)	0.82 (0.42–1.63)	1.08 (0.36–3.22)
	Female	106	85 (80.2)	0.577	0.888
Smoking history	Smoker	99	77 (77.8)	0.89 (0.45–1.78)	1.06 (0.36–3.14)
	Never-smoker	98	78 (79.6)	0.756	0.913
Pathological Stage	≥ II	56	35 (62.5)	0.29 (0.14–0.59)	0.38 (0.17–0.86)
	I	141	120 (85.1)	< 0.001	0.021
Pleural invasion	Present	45	32 (71.1)	0.58 (0.27–1.24)	1.85 (0.66–5.20)
	Absent	152	123 (80.9)	0.161	0.246
Lymphatic invasion	Present	26	15 (57.7)	0.30 (0.13–0.72)	0.63 (0.23–1.74)
	Absent	171	140 (81.9)	0.007	0.372
Vascular invasion	Present	59	40 (67.8)	0.42 (0.21–0.85)	0.56 (0.22–1.47)
	Absent	138	115 (83.3)	0.016	0.241
Grade	G2, 3	95	68 (71.6)	0.43 (0.21–0.88)	0.68 (0.28–1.61)
	G1	102	87 (85.3)	0.021	0.378
EGFR	Mutant	96	82 (85.4)	2.25 (1.10–4.59)	2.53 (1.06–6.04)
	Wild-type	101	73 (72.3)	0.027	0.036

OR odds ratio, *CI* confidence interval, *EGFR* epidermal growth factor receptor

CD44 Expression in CD44v9-Negative Cases

Among the CD44v9-negative group, we performed IHC using panCD44 antibody to confirm if CD44v9 expression was reduced through isoform switching or if the tumors were originally panCD44-negative. CD44v9-positive cases were also positive for panCD44, indicated by staining of the entire circumference of the membrane (electronic supplementary Figure 1B). Among the 75 CD44v9-negative cases, 29 (36.9%) were positive for panCD44 (electronic supplementary Table 2). We also analyzed survival among patients with advanced cancers categorized as CD44v9−/CD44−, CD44v9−/CD44+, and CD44v9+, respectively. The survival curves for the CD44v9−/CD44+ and CD44v9+ groups overlapped, while CD44v9−/CD44+ patients had a slightly poorer prognosis than CD44v9−/CD44− patients. Figure 3 shows representative cases with non-invasive CD44v9+/CD44+ areas and invasive CD44v9−/CD44+ areas.

DISCUSSION

To our knowledge, this is the first study to investigate the expression of CD44v9 in lung cancer. Our major findings are as follows: (1) CD44v9 was significantly associated with mutant *EGFR* (OR 2.53, 95% CI 1.06–6.04; $p = 0.036$) (Table 2) and early-stage tumors; and (2) DFS was slightly worse in patients with CD44v9 expression than in those without, in stage II and III–IV, although the difference was not significant due to the small sample size (Fig. 3).

Electronic supplementary Table 3 summarizes the studies reporting outcomes of various cancers with CD44v9 expression.[11–14,19–21] As shown in the table, several studies report that CD44v9 expression predicts poor prognosis of patients with advanced head and neck cancers, early gastric cancer, and bladder cancer.[12–14] In contrast, other studies found that reduced expression of CD44v9 is associated with lymph node metastasis and shorter survival of patients with squamous cell carcinoma of the tongue.[20] Furthermore, CD44v9 expression correlates with better prognosis of patients with high-grade serous ovarian cancer.[11] Therefore, these studies indicate that the significance of CD44v9 expression differs among certain cancers. In our study, DFS was slightly worse in patients with CD44v9 expression than in those without. Although the difference was not significant due to the small sample size, our results may support the clinical study targeting CD44v9 in advanced NSCLC.[16]

In addition, we also demonstrated that CD44v9−/CD44+ patients, i.e. patients with reduced CD44v9 expression or isoform switched CD44v9, also had a slightly poorer prognosis than those without CD44.

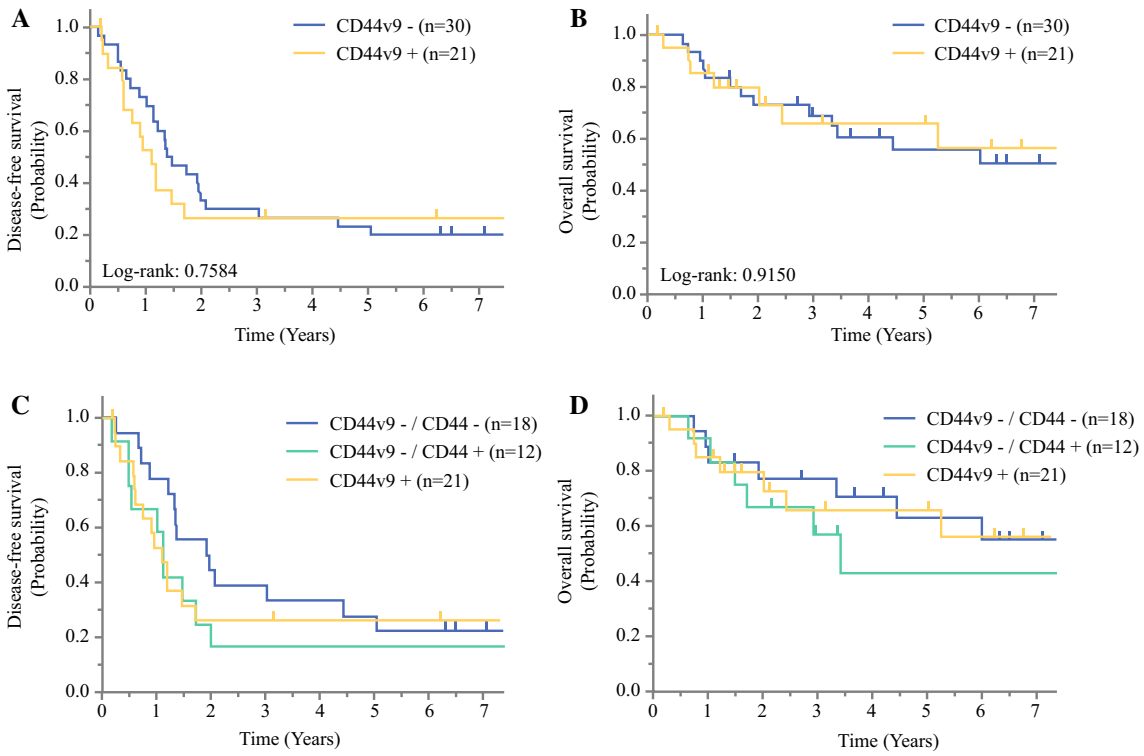

FIG. 2 Kaplan–Meier analysis of stage III–IV patients with lung adenocarcinoma according to CD44v9 and panCD44 expression. **a** Disease-free and **b** overall survival of patients according to CD44v9 expression. **c** Disease-free and **d** overall survival of patients according to panCD44 expression among those without CD44v9 expression

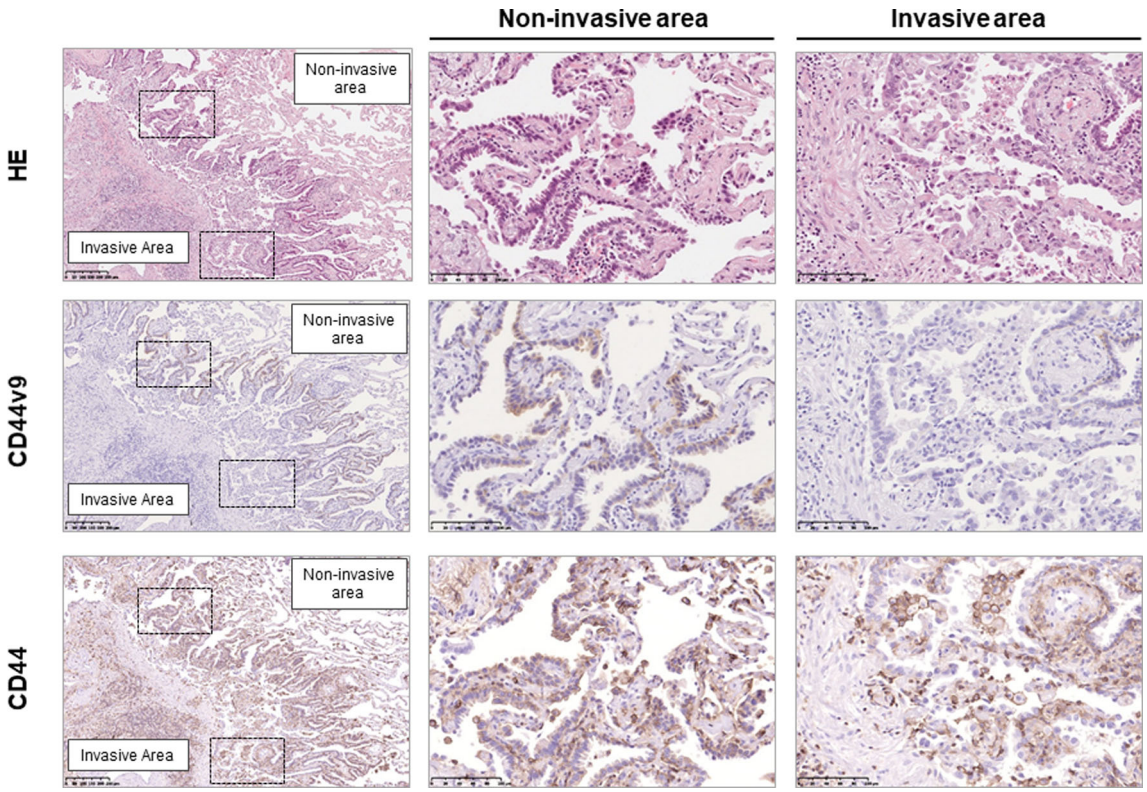

FIG. 3 Representative pathological image of lung adenocarcinoma showing invasive and noninvasive areas stained with HE, CD44v9, and panCD44. *HE* hematoxylin and eosin

Taken together, CD44v9 expression itself, as well as reduced CD44v9 expression, may contribute to tumors through different mechanisms, suggesting that CD44 may contribute to tumors via alteration of CD44v9 expression. First, CD44v9, which serves as a marker of CSCs, regulates the defense against ROS, resulting in tumorigenicity and drug resistance. Second, switching from a CD44v isoform to CD44 standard (CD44 s) triggers the induction of the epithelial-mesenchymal transition (EMT).

Previous reports have supported the second speculation that reduced expression of CD44v9 as a result of switching from CD44v9 to CD44 s induces EMT.[22] The switch from CD44v to CD44 s is significantly associated with the EMT and serves as a predictor of poor prognosis of patients with colorectal cancer.[23] Additionally, the switch from CD44v to CD44s is important for the EMT in breast and ovarian cancers.[11,22] Moreover, the ZEB1-driven increase in CD44 s expression, through repression of ESRP1, switches splicing of the *CD44* transcript encoding CD44s to generate the mRNA encoding CD44v in patients with NSCLC.[24] Although it is unclear whether CD44v9 is associated with the EMT, reduced CD44v9 expression may also modulate the malignant phenotype of lung adenocarcinoma, as shown in Fig. 3, demonstrating reduced CD44v9 expression in front of the invasive area.

Our data show that CD44v9 expression was significantly associated with mutant *EGFR* ($p = 0.036$). Although numerous studies focus on the association of certain cancers with CD44v,[9] one report examined the relationship between CD44v and *EGFR* mutation status in an NSCLC cell line and found that *EGFR* signaling induced by the *EGFR* mutation increases intracellular ROS levels and that CD44v plays an important role in redox adaptation of mutant *EGFR* NSCLC cells.[25] With regard to the association between CD44v9 and EMT, another report suggested that the upregulation of CD44s, in other words the isoform switch to CD44v to CD44s, occurs in *EGFR*-mutant lung cancer after acquisition of resistance to *EGFR* TKIs.[26] Therefore, CD44v9 has a potential biomarker to predict the emergence of acquired resistance to EGFR-TKI, and this should be elucidated in further studies. Together with these findings, our present data support the conclusion that CD44v9 plays a crucial role in *EGFR*-driven lung cancer.

LIMITATIONS

There are several limitations to our study. First, this was a retrospective study conducted using samples acquired at a single center, and the sample size of patients with advanced stage was too small to analyze. Second, we used the Allred score for the evaluation of CD44v9 expression, which must be validated by further studies.

CONCLUSION

We showed that CD44v9 expression was significantly associated with early-stage lung adenocarcinoma and mutant EGFR, and CD44v9 expression may play an important role in mutant EGFR tumors. In addition, CD44v9 expression itself, as well as reduced CD44v9 expression, may modulate the malignant phenotype of lung adenocarcinoma.

ACKNOWLEDGMENTS The authors thank Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

FUNDING This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

DISCLOSURE None.

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