Clinical Significance of Tumour CD44v and MIST1 Expression in Patients With Non-small-cell Lung Cancer

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Abstract. *Background:* Cancer stem cells (CSCs) are involved in cancer metastasis and relapse. Therefore, identification of CSC biomarkers might help determine the success of a treatment. In this study, we examined the expression of four CSC markers: Cluster of differentiation 44 variant (CD44v), leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), frizzled 7 (FZD7), and muscle, intestine and stomach expression 1 (MIST1), in cancer tissues of patients with non-small-cell lung cancer at >5 years after resection, and its clinical significance. *Patients and Methods:* We examined the expression of each CSC marker in 360 patients with NSCLC (n=360) who underwent curative resection by immunohistochemical analysis of tissue microarrays, and determined its relationship with survival. *Results:* High expression of MIST1 was related to better overall survival ($p<0.05$); high CD44v expression was associated with poor overall and recurrence-free survival ($p<0.001$ for both) and thus, CD44v was defined as an independent prognostic factor ($p<0.05$), according to a multivariate analysis. *Conclusion:* Tumoral CD44v expression might be a useful prognostic marker for patients after curative resection of NSCLC.

Lung cancer is the most frequent malignant tumour with the highest mortality rate worldwide (1). Non-small-cell lung cancer

(NSCLC) accounts for 80-85% of all lung cancer and its best treatment is surgical intervention (2). However, even with curative resection, a 5-year survival rate of 64% is inadequate (3). Therefore, an improved treatment outcome, such as a treatment individualisation based on biomarkers, is necessary.

In 1997, cancer was confirmed to originate from a small number of cells, which have the self-replication and diversification characteristics of stem cells (4), cancer stem cells (CSCs) and then by 2000, CSCs were found in various types of cancer. Previous studies have reported that CSCs are resistant to various treatments, such as chemotherapy and radiation (5-8). Although CSCs are associated with patient survival, there is insufficient information regarding identification of CSC markers that are useful in predicting prognosis. Therefore, based on the data from The Cancer Genome Atlas and many previous reports on CSC markers, we studied four possible biomarkers of NSCLC: Cluster of differentiation 44 variant (CD44v), leucine-rich-repeat-containing g-protein-coupled receptor 5 (LGR5), frizzled-7 (FZD7), and muscle, intestine and stomach expression 1 (MIST1).

CD44, a non-kinase transmembrane glycoprotein, plays a role in cancer development and progression (9, 10) and its expression in NSCLC is not clearly related to patient survival (11-18).

LGR5 is a target gene of the WNT signalling pathway and an intestinal epithelial stem cell marker (19). Studies have reported that LGR5 overexpression is associated with the prognosis of several malignant tumours (20-26). However, its association with lung cancer has not been determined.

MIST1 is a transcriptional factor of the basic helix-loop-helix family and a CSC marker. Its mRNA was first observed in the developing lung bud during mouse embryogenesis (27) and then on mammalian lung cells. However, the clinical significance of MIST1 expression in lung cancer tissue has not been clarified.

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FZD receptors, FZD1 to FZD10, can respond to WNT proteins in the presence of WNT co-receptors to activate WNT pathways. FZD7 is the most commonly up-regulated FZD molecule in a variety of cancer types, including lung cancer (28, 29), and its mRNA overexpression was upregulated in lung adenocarcinoma cell lines, promoting cell viability and survival. In addition, FZD7 was reported to play an important role in stem cell biology and in cancer development and progression. However, its clinical significance in lung cancer has not been determined (30, 31).

With the evolution of cigarette composition, smoking habits, environmental, and lung cancer screening methods, the histological distribution of NSCLC is changing (32).

In this study, we investigated the clinical significance of the expression of these four CSC markers in cancer tissues of patients who had undergone curatively resected NSCLC.

Patients and Methods

Patients. This study was approved by the Research Ethics Committee of the Kanagawa Cancer Center prior to initiating the study (approval number: Epidemiological Study-2019-133). A total of 396 patients diagnosed with lung cancer who underwent surgery between August 2011 and August 2015 at the Kanagawa Cancer Center were selected from the clinical database. The inclusion criteria were: i) A pathological diagnosis of NSCLC, and ii) undergoing curative resection (R0). The exclusion criteria were: i) Death prior to discharge, ii) pathological diagnosis of stage IV, and iii) receipt of neoadjuvant chemotherapy/radiotherapy.

The tumour histology and the pathological stage were determined using the World Health Organization classification (33) and the seventh edition of the TNM classification for lung cancer (34), respectively.

Overall survival (OS) was measured from the operative day to death, and recurrence-free survival (RFS) from the operative day to tumour recurrence or death by any cause.

Immunohistochemical analysis using tissue microarray. Blocks containing formalin-fixed paraffin-embedded lung cancer tissues were selected by pathologists. The slides sectioned from the blocks were stained with haematoxylin and eosin (H&E) and the central and peripheral parts of lung cancer tissues as well as the adjacent normal tissue were marked by a pathologist. Next, two central and two peripheral cores were collected from the marked areas of each block by using tissue cylinders. The four tissue sections were deparaffinised and soaked in 10 mM sodium citrate buffer at 121°C for 20 min. After blocking with 5% skim milk in phosphate buffered saline with Tween® 20 (Takara Bio, Shiga, Japan), the sections were incubated overnight with anti-CD44v (1:500), anti-LGR5 (1:25), anti-MIST1 (1:100), and anti-FZD7 (1:125) primary antibodies (all Abcam PLC, Cambridge, UK) at 4°C. A peroxidase-labelled polymer (DAKO EnVision™ + System, HRP; DAKO, Glostrup, Denmark) was used to detect the signals. All sections were counterstained with H&E.

Immunohistochemical evaluation of CD44v immunostaining was performed based on the report published by Hirata *et al.* (11). Scores were assigned as a percentage of positive core. The mean percentage for four cores was considered representative of one tumour. CD44v expression was considered low or high, depending

Table I. Clinicopathological patient.

Characteristic	Value
Gender	
Male	203 (56.4%)
Female	157 (43.6%)
Age	
Median (range)	68 (35-90)
<69 Years	187 (51.9%)
≥69 Years	173 (48.1%)
Smoking habit, n (%)	
Non smoker	140 (38.9%)
Smoker	220 (61.1%)
Serum CEA	
≤5.0 ng/ml	284 (78.9%)
>5.0 ng/ml	76 (21.1%)
Surgical procedure, n (%)	
Lobectomy or more	286 (79.4%)
Segmentectomy	26 (7.2%)
Wedge resection	48 (13.3%)
Histological type, n (%)	
Adenocarcinoma	290 (80.6%)
Squamous cell carcinoma	36 (10.0%)
Large-cell carcinoma	15 (4.2%)
Other	19 (5.3%)
p-Stage*, n (%)	
I	273 (75.8%)
II	52 (14.4%)
III	35 (9.7%)
pT Category, n (%)	
1	176 (48.9%)
2	163 (45.3%)
3	21 (5.9%)
4	0 (0.0%)
pN Category, n (%)	
0	298 (82.8%)
1	30 (8.3%)
2	32 (8.9%)
Lymphatic invasion, n (%)	
–	289 (80.3%)
+	71 (19.7%)
Vessel invasion, n (%)	
No	249 (69.2%)
Yes	111 (30.8%)
Pleural invasion, n (%)	
No	277 (76.9%)
Yes	83 (23.1%)
Adjuvant chemotherapy, n (%)	
No	282 (78.3%)
Yes	78 (21.7%)

*According to the seventh edition of the TNM classification for lung cancer (34).

on whether the percentage of positively stained cells was less than 20% or higher, respectively. Immunohistochemical evaluation of LGR5, MIST1, and FZD7 expression was performed according to a modified immunoreactivity scoring system (IRS). The maximum intensity of positively immunostained tumour cells was classified as absent (score 0), weak (score 1), moderate (score 2), and strong

Figure 1. Representation high and low immunohistochemical expression of cluster of differentiation 44 variant (CD44v), leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), muscle, intestine and stomach expression 1 (MIST1) and frizzled 7 (FZD7) in non-small-cell lung cancer tissues. CD44v was located in the cell membrane, whereas LGR5, MIST1, and FZD7 were located in the cytoplasm and nucleus $\times 400$.

(score 3). The percentage of positively immunostained tumour cells was divided into four scores, namely 0, 1, 2 and 3, based on the marker-specific approach described by Grote *et al.* (35). Therefore, samples with 0% tumour cells immunostained for LGR5, MIST1, and FZD7 was scored as 0; tumour cell immunostaining of 0.1-19% for LGR5, 0.1-1% MIST1, and 0.1-1% FZD7 were scored as 1; immunostaining of 20-49% for LGR5, 2-10% for MIST1, and 2-10% for FZD7 were scored as 2; and a score of 3 for $\geq 50\%$ LGR5, $\geq 11\%$ MIST1 and $\geq 11\%$ FZD7 immunostained tumour cells were scored as 3. The scores percentage staining and intensity were added, resulting in IRS values of 0 to 6. IRS values of between 0 to 4 were defined as representing low immunoreactivity and those between 5 and 6 as high immunoreactivity. All arrays were reviewed by two unblinded pathologists. Discordant cases were reviewed and discussed until a consensus was reached.

Statistical analysis. Survival curves were constructed according to the Kaplan–Meier method to evaluate the correlations between the expression of each CSC marker and the survival rate. The differences in survival between patients with high and those with low expression levels were analysed using the log-rank test. The relationships between expression levels of CSC markers and clinicopathological factors were assessed using Pearson's chi-squared test. To identify the factors involved in OS and RFS, the Cox proportional hazards model was used. The multivariate analysis included influencing factors with a value of $p < 0.1$ in the univariate analysis. Statistical analyses of the results were performed using the SPSS 23.0 statistical package (IBM Corp., Armonk, NY, USA). For all tests, two-sided p -values of less than 0.05 were considered statistically significant.

Results

Characteristics of the study patients. The characteristics of the study patients are summarised in Table I. A total of 360 out of 396 patients (91.1 %) were included. The median follow-up period was 66.0 months. Regarding the histological cancer types: 290 cases presented adenocarcinoma, 36 squamous cell carcinoma, 15 large-cell carcinoma, and 19 other non-small-cell carcinomas, including carcinoid tumour ($n=1$), and large cell neuroendocrine ($n=7$), adenosquamous ($n=5$), pleomorphic ($n=5$), and unclassified ($n=1$) carcinomas. Based on the pathological stage, disease in 273, 52, and 35 patients corresponded to stage I, II, and III, respectively.

CD44v, LGR5, FZD7, and MIST1 expression in NSCLC. Figure 1 shows representative expression of each CSC marker at low and high levels in cancer tissue. Rates of high expression of CD44v, LGR5, FZD7, and MIST1 were 15.2%, 67.7%, 16.2%, and 23.9%, respectively.

OS and RFS according to CD44v, LGR5, MIST1, and FZD7 expression levels. High CD44v expression was significantly correlated with worse OS and RFS ($p < 0.001$) when compared to low CD44v expression. The 5-year OS rates in patients with high and low CD44v expression were 64.3% and 83.8%, respectively (Figure 2A), whereas the corresponding 5-year RFS rates were 48.9% and 74.8%, respectively (Figure 3A).

Figure 2. Overall survival according to cluster of differentiation 44 variant (CD44v) (A), leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) (B), muscle, intestine and stomach expression 1 (MIST1) (C), and frizzled 7 (FZD7) (D) expression. High CD44v expression was significantly associated with a poor OS compared to that of the low CD44v expression group ($p<0.001$). Low MIST1 expression was significantly poorer than that of the high MIST1 group ($p=0.031$). There was no significant difference in OS depending on the expression levels of LGR5 and FZD2.

However, low MIST1 expression was significantly associated with worse OS ($p=0.031$) when compared to patients with high MIST1 expression (Figure 2C). The 5-year OS rates in patients with high and low MIST1 expression levels were 88.2% and 78.2%, respectively. Analyses of LGR5 and FZD2 levels did not indicate significant differences in OS or RFS dependent on the expression levels.

Relationship between CD44v, LGR5, MIST1, and FZD7 expression levels and clinicopathological factors. Relationships between expression levels of these four CSC markers and clinicopathological factors are shown in Tables II and III. High expression of CD44v was significantly correlated with male sex, a smoking habit, histological type (squamous cell carcinoma), pathological T category (T2 or T3), and vessel and pleural invasion. High expression of MIST1 was significantly correlated with lower serum carcinoembryonic antigen (CEA) level, pathological stage I, and pathological category N0. High expression of LGR5 was

significantly correlated with vessel invasion. However, no significant correlation between FZD7 expression levels and clinicopathological factors was noted.

Univariate and multivariate analyses of OS. According to the univariate analysis, sex, smoking habit, serum CEA level, histological type, pathological stage, lymphatic invasion, vessel invasion, CD44v expression, and MIST1 expression were selected as significant factors for OS. On the other hand, the multivariate analysis of OS indicated that serum CEA level, histological type, pathological stage, and CD44v positivity were independent factors for OS (Table IV).

Univariate and multivariate analyses of RFS. The univariate analysis of RFS revealed that sex, serum CEA level, surgical procedure, histological type, pathological stage, lymphatic invasion, vessel invasion, adjuvant chemotherapy, and CD44v expression were significant factors for RFS. However, only serum CEA level, pathological stage, histological type,

Figure 3. Recurrence-free survival (RFS) according to cluster of differentiation 44 variant (CD44v) (A), leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) (B), muscle, intestine and stomach expression 1 (MIST1) (C), and frizzled 7 (FZD7) (D) expression. RFS of the group with high CD44v expression was significantly poorer than that with low CD44v expression. There was no significant difference in RFS depending on the expression levels of LGR5, MIST1 and FZD2.

lymphatic invasion, and CD44v expression were significant independent factors for RFS, based on the multivariate analysis of RFS (Table V).

Discussion

In the present study, we investigated whether the expression of CSC markers: CD44v, LGR5, FZD7, and MIST1 in cancer tissues of patients with NSCLC had clinical significance. Our results indicated that high expression of CD44v and low expression of MIST1 were significantly associated with poor outcomes and that CD44v expression was a useful prognostic factor in patients with NSCLC.

Several studies reported that high expression of CD44v was significantly associated with poor outcomes (11-13). However, others indicated the opposite (14, 15). Thus, the clinical significance of CD44v expression in NSCLC tissue was not clearly defined. A possible reason for this discrepancy might be that the conditions of these previous

studies were different, including the ratio of the histological type, tumour stage, and ethnicity, and that those different conditions might lead to different results when studying the relationship between CD44v expression and survival. In particular, the ratio of histological type was historically different. With the evolution of cigarette composition, smoking habits, environmental exposure, and lung cancer screening methods, the histological distribution of NSCLC is changing.

Two meta-analyses about the association between CD44v and NSCLC reported that high expression of CD44v was a poor prognostic factor (16, 17). However, the total number of patients in each study was smaller than that in this study, and several aspects were different.

The present study included the largest number of patients investigated for the true association between CD44v expression and survival compared to previous ones. Moreover, we used the latest samples and data with more than 5 years prognostic information.

Table II. Relationship between Cluster of differentiation 44 variant (CD44v) and muscle, intestine, and stomach expression 1 (MIST1) expression and clinicopathological parameters.

Characteristic	CD44v, n (%)			MIST1, n (%)		
	Low (n=305)	High (n=55)	p-Value	Low (n=274)	High (n=86)	p-Value
Gender						
Male	155 (76.4%)	48 (23.6%)	<0.001	158 (77.8%)	45 (22.2%)	0.384
Female	150 (95.5%)	7 (4.5%)		116 (91.7%)	41 (26.1%)	
Age						
<69 Years	163 (87.2%)	24 (12.8%)	0.108	144 (77.0%)	43 (23.0%)	0.679
≥69 Years	142 (82.1%)	31 (17.9%)		130 (75.1%)	43 (24.9%)	
Smoking habit						
Non smoker	137 (97.9%)	3 (2.1%)	<0.001	100 (71.4%)	40 (28.6%)	0.096
Smoker	168 (76.4%)	52 (23.6%)		174 (79.1%)	46 (20.9%)	
Serum CEA						
≤5.0 ng/ml	244 (85.9%)	40 (14.1%)	0.224	207 (72.9%)	77 (27.1%)	0.006
>5.0 ng/ml	61 (80.3%)	15 (19.7%)		67 (88.2%)	9 (11.8%)	
Surgical procedure						
Lobectomy or more	240 (83.9%)	46 (16.1%)	0.403	220 (76.9%)	66 (23.1%)	0.478
Sublobar resection	65 (87.8%)	9 (12.2%)		54 (73.0%)	20 (27.0%)	
Histological type						
Overall			<0.001			0.488
Adenocarcinoma	276 (95.2%)	14 (4.8%)	<0.001 vs. other	217 (74.8%)	73 (25.2%)	
Squamous cell carcinoma	6 (16.7%)	30 (83.3%)		31 (86.1%)	5 (13.9%)	
Large-cell carcinoma	9 (60.0%)	6 (40.0%)		12 (80.0%)	3 (20.0%)	
Other	14 (73.7%)	5 (26.3%)		14 (73.7%)	5 (26.3%)	
p-Stage*						
Overall			0.843			0.001
I	233 (85.3%)	40 (14.7%)	0.608 vs. II, III	195 (71.4%)	78 (28.6%)	<0.001 vs. II, III
II	43 (82.7%)	9 (17.3%)		47 (90.4%)	5 (9.6%)	
III	29 (85.3%)	5 (14.7%)		32 (91.4%)	3 (8.6%)	
pT Category						
Overall			0.030			0.151
1	158 (89.8%)	18 (10.2%)	0.009 vs. 2, 3	128 (72.7%)	48 (27.3%)	0.141 vs. 2, 3
2	131 (80.4%)	32 (19.6%)		127 (77.9%)	36 (22.1%)	
3	16 (76.2%)	5 (23.8%)		19 (90.5%)	2 (9.5%)	
pN Category						
Overall			0.817			0.006
0	254 (85.2%)	44 (14.8%)	0.553 vs. 1, 2	217 (72.8%)	81 (27.2%)	0.001 vs. 1, 2
1	25 (83.3%)	5 (16.7%)		28 (93.3%)	2 (6.7%)	
2	26 (81.3%)	6 (18.8%)		29 (90.6%)	3 (9.4%)	
Lymphatic invasion						
No	247 (85.5%)	42 (14.5%)	0.428	217 (75.1%)	72 (24.9%)	0.358
Yes	58 (81.7%)	13 (18.3%)		57 (80.3%)	14 (19.7%)	
Vessel invasion						
No	223 (89.6%)	26 (10.4%)	<0.001	187 (75.1%)	62 (24.9%)	0.501
Yes	82 (73.9%)	29 (26.1%)		87 (78.4%)	24 (21.6%)	
Pleural invasion						
No	241 (87.0%)	36 (13.0%)	0.028	211 (76.2%)	66 (23.8%)	0.960
Yes	64 (77.1%)	19 (22.9%)		63 (75.9%)	20 (24.1%)	
Adjuvant chemotherapy						
No	238 (84.4%)	44 (15.6%)	0.744	211 (74.8%)	71 (25.2%)	0.276
Yes	67 (85.9%)	11 (14.1%)		63 (80.8%)	15 (19.2%)	

*According to the seventh edition of the TNM classification for lung cancer (34). Statistically significant p-values are shown in bold.

Moreover, this study is the first to report the association between MIST1 and NSCLC. We demonstrated that the high expression of MIST1 was significantly associated with better OS and correlated with better prognostic factors, including

low CEA level, pathological stage I, and pN0 category. A previous study showed that MIST1 functioned as a tumour suppressor in mice and that MIST1 protein and mRNA expression were both down-regulated in a pancreatic cancer

Table III. Relationship between leucin-rich-repeat-containing *g*-protein-coupled receptor 5 (*LGR5*) and *frizzled-7* (*FZD7*) expression and clinicopathological parameters.

Characteristic	LGR5, n (%)			FZD7, n (%)		
	Low (n=115)	High (n=242)	<i>p</i> -Value	Low (n=300)	High (n=58)	<i>p</i> -Value
Gender						
Male	67 (33.3%)	134 (66.7%)	0.607	169 (84.1%)	32 (15.9%)	0.870
Female	48 (30.8%)	108 (69.2%)		131 (83.4%)	26 (16.6%)	
Age						
<69 Years	52 (28.1%)	133 (71.9%)	0.085	156 (83.9%)	30 (16.1%)	0.969
≥69 Years	63 (36.6%)	109 (63.4%)		144 (83.7%)	28 (16.3%)	
Smoking habit						
Non smoker	41 (29.5%)	98 (70.5%)	0.380	116 (82.9%)	24 (17.1%)	0.698
Smoker	74 (33.9%)	144 (66.1%)		184 (84.4%)	34 (15.6%)	
Serum CEA						
≤5.0 (ng/ml)	89 (31.4%)	194 (68.6%)	0.546	239 (84.2%)	45 (15.8%)	0.720
<5.0 (ng/ml)	26 (35.1%)	48 (64.9%)		61 (82.4%)	13 (17.6%)	
Surgical procedure						
Lobectomy or more	91 (32.2%)	192 (67.8%)	0.964	235 (82.7%)	49 (17.3%)	0.290
Sublobar resection	24 (32.4%)	50 (67.6%)		65 (87.8%)	9 (12.2%)	
Histological type						
Adenocarcinoma	89 (31.0%)	198 (69.0%)	0.560	239 (83.0%)	49 (17.0%)	0.732
Squamous cell carcinoma	15 (41.7%)	21 (58.3%)		31 (86.1%)	5 (13.9%)	
Large-cell carcinoma	4 (26.7%)	11 (73.3%)		14 (93.3%)	1 (6.7%)	
Other	7 (36.8%)	12 (63.2%)		16 (84.2%)	3 (15.8%)	
p-Stage*						
Overall			0.826			0.157
I	86 (31.6%)	186 (68.4%)	0.667 vs. II, III	227 (83.2%)	46 (16.8%)	0.551 vs. II, II
II	18 (36.0%)	32 (64.0%)		46 (92.0%)	4 (8.0%)	
III	11 (31.4%)	24 (68.6%)		27 (77.1%)	8 (22.9%)	
pT Category						
Overall			0.066			0.745
1	51 (29.3%)	123 (70.7%)	0.252 vs. 2, 3	145 (82.9%)	30 (17.1%)	0.636 vs. 2, 3
2	53 (32.5%)	110 (67.5%)		139 (85.3%)	24 (14.7%)	
3	11 (55.0%)	9 (45.0%)		16 (80.0%)	4 (20.0%)	
pN Category						
Overall			0.852			0.474
0	96 (32.4%)	200 (67.6%)	0.845 vs. 1, 2	249 (83.8%)	48 (16.2%)	0.964 vs. 1, 2
1	10 (34.5%)	19 (65.5%)		26 (89.7%)	3 (10.3%)	
2	9 (28.1%)	23 (71.9%)		25 (78.1%)	7 (21.9%)	
Lymphatic invasion						
No	96 (33.4%)	191 (66.6%)	0.311	242 (84.0%)	46 (16.0%)	0.812
Yes	19 (27.1%)	51 (72.9%)		58 (82.9%)	12 (17.1%)	
Vessel invasion						
No	88 (35.6%)	159 (64.4%)	0.039	208 (83.9%)	40 (16.1%)	0.956
Yes	27 (24.5%)	83 (75.5%)		92 (83.6%)	18 (16.4%)	
Pleural invasion						
No	92 (33.5%)	183 (66.5%)	0.358	228 (82.6%)	48 (17.4%)	0.262
Yes	23 (28.0%)	59 (72.0%)		72 (87.8%)	10 (12.2%)	
Adjuvant chemotherapy						
No	90 (31.1%)	189 (67.7%)	0.972	238 (85.0%)	42 (15.0%)	0.243
Yes	25 (32.1%)	53 (67.9%)		62 (79.5%)	16 (20.5%)	

*According to the seventh edition of the TNM classification for lung cancer (34).

cell line (36). Moreover, overexpression of *MIST1* reversed the epithelial–mesenchymal transition by down-regulating snail family transcriptional repressor 1 (*SNAIL*) and up-regulating E-cadherin (36).

Regarding LGR5, two studies showed that high expression of LGR5 was significantly associated with poor prognosis in patients with NSCLC and lung adenocarcinoma (24, 25). However, the LGR5 expression level was not associated with

Table IV. Uni- and multivariate analyses of overall survival in patients with non-small-cell lung cancer (n=360).

Factor	Comparison	Univariate		Multivariate	
		HR (95% CI)	p-Value	HR (95% CI)	p-Value
Gender	Male vs. female	0.479 (0.291-0.786)	0.004	0.782 (0.365-1.674)	0.527
Age	<69 vs. ≥69 Years	1.349 (0.859-2.119)	0.193		
Smoking habit	No vs. yes	1.965 (1.179-3.275)	0.010	0.870 (0.392-1.932)	0.732
CEA	≤5.0 vs. <5.0 ng/ml	3.647 (2.318-5.740)	<0.001	2.939 (1.828-4.725)	<0.001
Surgical procedure	Sublobar resection vs. lobectomy or more	1.203 (0.673-2.149)	0.532		
Histological type	Adenocarcinoma	Reference		Reference	
	Squamous cell carcinoma	2.965 (1.629-5.397)	<0.001	1.686 (0.742-3.834)	0.212
	Large-cell carcinoma	3.129 (1.336-7.331)	0.009	1.924 (0.779-4.752)	0.156
	Other	4.893 (2.464-9.715)	<0.001	4.077 (1.908-8.711)	<0.001
Pathological stage*	I vs. II, III	3.031 (1.927-4.765)	<0.001	2.064 (1.179-3.613)	0.011
Lymphatic invasion	No vs. yes	2.597 (1.623-4.156)	<0.001	1.354 (0.784-2.338)	0.277
Vessel invasion	No vs. yes	2.464 (1.569-3.868)	<0.001	1.084 (0.644-1.824)	0.762
Adjuvant chemotherapy	No vs. yes	1.034 (0.603-1.774)	0.903		
CD44v expression	Low vs. high	2.974 (1.822-4.857)	<0.001	2.104 (1.078-4.106)	0.029
LGR5 expression	Low vs. high	0.834 (0.517-1.344)	0.456		
FZD7 expression	Low vs. high	1.202 (0.672-2.150)	0.536		
MIST1 expression	Low vs. high	0.502 (0.265-0.952)	0.035	0.753 (0.386-1.471)	0.407

Cluster of differentiation 44 variant (CD44v), leucine-rich-repeat-containing g-protein-coupled receptor 5 (LGR5), frizzled-7 (FZD7), and muscle, and intestine, and stomach expression 1 (MIST1). *According to the seventh edition of the TNM classification for lung cancer (34). Statistically significant p-values are shown in bold.

Table V. Uni- and multivariate analyses of recurrence-free survival in patients with non-small-cell lung cancer (n=360).

Factor	Comparison	Univariate		Multivariate	
		HR (95% CI)	p-Value	HR (95% CI)	p-Value
Gender	Male vs. female	0.642 (0.434-0.949)	0.026	0.885 (0.489-1.602)	0.687
Age	<69 vs. ≥69 Years	0.923 (0.635-1.343)	0.667		
Smoking habit	No vs. yes	1.490 (0.997-2.225)	0.052	0.856 (0.458-1.599)	0.626
CEA	≤5.0 vs. <5.0 ng/ml	2.524 (1.706-3.734)	<0.001	1.673 (1.107-2.527)	0.015
Surgical procedure	Sublobar resection vs. lobectomy or more	1.820 (1.055-3.139)	0.031	0.714 (0.485-1.042)	0.714
Histological type	Adenocarcinoma	Reference		Reference	
	Squamous cell carcinoma	1.947 (1.135-3.339)	0.016	1.189 (0.586-2.410)	0.632
	Large-cell carcinoma	2.243 (1.034-4.867)	0.041	1.446 (0.632-3.307)	0.382
	Other	3.208 (1.701-6.052)	<0.001	2.165 (1.086-4.317)	0.028
Pathological stage*	I vs. II, III	4.037 (2.773-5.877)	<0.001	2.335 (1.424-3.828)	0.001
Lymphatic invasion	No vs. yes	3.330 (2.267-4.891)	<0.001	1.613 (1.028-2.531)	0.038
Vessel invasion	No vs. yes	3.194 (2.195-4.648)	<0.001	1.514 (0.970-2.361)	0.068
Adjuvant chemotherapy	No vs. yes	2.000 (1.346-2.972)	<0.001	1.236 (0.796-1.920)	0.345
CD44v expression	Low vs. high	2.519 (1.654-3.836)	<0.001	2.051 (1.176-3.578)	0.011
LGR5 expression	Low vs. high	1.158 (0.763-1.757)	0.491		
FZD7 expression	Low vs. high	1.334 (0.835-2.130)	0.228		
MIST1 expression	Low vs. high	0.614 (0.374-1.007)	0.053	0.872 (0.516-1.473)	0.608

CD44v Cluster of differentiation 44 variant; LGR5: leucine-rich-repeat-containing G-protein-coupled receptor 5; FZD7: frizzled-7; MIST1: muscle, and intestine, and stomach expression 1. *According to the seventh edition of the TNM classification for lung cancer (34). Statistically significant p-values are shown in bold.

survival in our patients. Previous examination of the relationship between LGR5 expression and clinicopathological factors indicated an association between pathological stage and tumour size (24-26). However, in our study, although high expression of

LGR5 was associated with vessel invasion, expression of LGR5 was not associated with pathological stage and tumour size.

Some studies indicated that expression of *FZD7* mRNA was up-regulated in lung adenocarcinoma cell lines and

promoted cell viability and survival (30, 31). However, in this study, the expression levels of FZD7 were not significantly associated with survival even when in the adenocarcinoma subgroup analysis.

Our study had several limitations. Firstly, although this study was conducted using a relatively large number of patients with NSCLC (n=360), it was a single-centre, retrospective study. To obtain more reliable results regarding the clinical significance of CSC markers, a multicentre study with a larger number of cases is necessary. Secondly, in this study, we examined the CSC marker levels at the central and peripheral regions of each NSCLC tissue by immunostaining; however, considering tumour heterogeneity, the expression levels of the CSC markers at these four regions might not be representative of the whole tumour.

In conclusion, the CD44v level in tumour tissues might be a useful clinical prognostic marker for patients undergo curatively resected NSCLC.

Conflicts of Interest

All Authors have no conflicts of interest or financial ties to disclose.

Authors' Contributions

Concept and study design were by Oshima T, Nagashima T, Hiroshima Y, and Miyagi Y. Data collection and literature searches were conducted by Nagashima T, and Ito H. Data analysis and interpretation were performed by Nagashima T, Oshima T, Hiroshima Y, and Miyagi Y. Data interpretation was carried out by all investigators. The article and figures were drafted by Nagashima T and Oshima T. Finally, this article was revised and approved by all investigators.

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