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Prognostic role of extracellular vesicles in squamous cell carcinoma of the lung

Hyo Jung An[1], Min Hye Kim[2], Sung Hwan Kim[3,4,5], Gyeong-Won Lee[3,4,6] & Dae Hyun Song[1,3,4]

1 Department of Pathology, Gyeongsang National University Changwon Hospital, Changwon, South Korea
2 Department of Pathology, Gyeongsang National University Hospital, Jinju, South Korea
3 Gyeongsang National University School of Medicine, Jinju, South Korea
4 Gyeongsang Institute of Health Science, Jinju, South Korea
5 Departments of Thoracic and Cardiovascular Surgery, Gyeongsang National University Changwon Hospital, Changwon, South Korea
6 Department of Internal Medicine, Gyeongsang National University Hospital, Jinju, South Korea

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Amphisome; exosome; extracellular vesicles; lung; squamous cell carcinoma.

Correspondence
Dae Hyun Song, Department of Pathology, Gyeongsang National University School of Medicine, 15 Jinju-daero 816 Beon-gil, Jinju 660-751, South Korea.
Tel: +82 55 214 3150
Fax: +82 55 214 3174
Email: golgy@hanmail.net

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Abstract

Background: Research on diagnosing recurrent non-small cell lung cancer (NSCLC) and applying target gene treatment using exosomes in a less invasive way is very important. Recently, however, it has been argued that exosomes do not contain double-stranded DNA (dsDNA) or histones. In this study, we describe the expression of extracellular vesicle (EV) markers in specimens from squamous cell carcinoma (SCC) of the lung and analyze their relationship with the prognosis of patients.

Methods: Clinical and pathological data were obtained from 96 patients who had undergone surgery for SCC of the lung. Tissue microarray blocks were made using representative paraffin blocks of samples from patients with SCC of the lung. Two pathologists graded the intensity of CD63, CD9, LC3A/B, P62, and ANXA1 expression as high or low expression. In addition, the authors designated the combined expression of these five independent markers as "positive EV expression" in this article.

Results: SCCs with low CD63 and SCCs with low EV expression showed unfavorable disease-free survival (DFS) (P-value = 0.037 and 0.006, respectively) in the survival analysis. The Kaplan-Meier survival curve confirmed that the low EV expression showed a statistically significant relationship with unfavorable DFS (P-value = 0.004). There were no statistically significant differences in DFS and disease-specific survival in each low and high expression group for CD9, LC3A/B, ANXA1, and P62 in the Cox regression analysis.

Conclusions: As EV expression was related to the prognosis of lung SCC patients, a broader approach using different extracellular vesicles rather than a conventional exosome-dependent one is needed.

Key points

Significant findings of the study: The combined expression of CD9, CD63, LC3A/B, ANXA1, and P62 as a marker for extracellular vesicles is effective in predicting the prognosis of patients with squamous cell carcinoma of the lung.

What this study adds: A broader approach using different extracellular vesicles rather than conventional exosome-dependent methods is required when diagnosing and treating lung cancer patients.

Introduction

Research on how extracellular vesicles (EVs) shed from cancer cells affect tumorigenesis has been performed.[1, 2] In particular, strategies to diagnose recurrent non-small cell lung cancer (NSCLC) and to apply target gene treatment using EVs are less invasive than percutaneous core needle biopsies. EVs are very small structures of less than 1000 nm

in size that are composed of a lipid bilayer and internal cargo and lipids. EVs are secreted from any kind of cell,[3] and they contain several kinds of genetic material, including microRNAs, other noncoding RNAs, and DNA that might be useful for either diagnosing or treating cancers. EVs are generally categorized by size: exosomes, between 30 and 100 nm; microvesicles, between 100 and 1000 nm; and apoptotic bodies, over 1000 nm.[4, 5] Jeppesen and colleagues established new nomenclature for extracellular vesicles and proposed markers of intracellular precursors of EVs: CD63 and CD9 for multivesicular endosomes (MVEs), P62, and LC3A/B for amphisomes, and ANXA1 for a novel marker for microvesicles.[6, 7] To investigate the prognostic role of EVs in pulmonary SCC patients, each EV marker was analyzed on a pulmonary SCC specimen using immunohistochemical staining. We designated the combined expression of these five immunohistochemical markers as "positive EV expression" in this article. This study aims to confirm the previous in vitro results of Jeppesen *et al.*[6] and to suggest a broad approach using different extracellular vesicles for patients with SCC of the lung.

Methods

Patients and clinicopathological data

A total of 96 patients who had undergone surgery for SCC of the lung between January 2002 and December 2009 at the Gyeongsang National University Hospital, Jinju, South Korea, were enrolled for the study. Representative hematoxylin and eosin (H&E)-stained slides from the 96 consecutive patients were re-examined by two pathologists. Electronic medical records were reviewed, and the clinical and pathological data, including age, sex, smoking history, surgery history, pathologic differentiation, TNM stage, T stage, N stage, disease-free survival (DFS), and disease-specific survival (DSS), were obtained. The stages of lung cancer were determined according to the eighth edition guidelines of the American Joint Committee on Cancer (AJCC). This study was approved by the institutional review board of the Gyeongsang National University Hospital (GNUH-2020-04-005).

Tissue microarray construction and immunohistochemistry

Representative H&E-stained glass slides containing intratumoral lesions were examined. A core (3 mm in size) was collected from the invasive tumor front of each representative paraffin block and transplanted into the recipient tumor microarray (TMA) blocks. Immunohistochemical staining was carried out using an automated immunostainer (Benchmark Ultra, Ventana Medical Systems Inc., Tucson, AZ, USA) with monoclonal antibodies: anti-CD9 antibody at a dilution of 1:50 (ab2215, Abcam, Cambridge, MA, USA, anti-CD63 antibody at a dilution of 1:500 (ab134045, Abcam, Cambridge, MA, USA), anti-LC3A/B antibody at a dilution of 1:100 (ab128025, Abcam, Cambridge, MA, USA), anti-P62 antibody at a dilution of 1:2000 (ab56416, Abcam, Cambridge, MA, USA), and anti-ANXA1 antibody at a dilution of 1:100 (ab33061, Abcam, Cambridge, MA, USA).

EV expression

The pattern of immunohistochemical staining was evaluated: nuclear expression for CD63, the membranous expression for CD9, the nuclear and cytoplasmic expression for P62 and LC3AB, and cytoplasmic expression for ANXA1 in the tumor cells. The expression patterns of these markers were compared with each clinicopathological characteristic, including age, sex, smoking and surgical history, pathological differentiation, T stage, and N stage (Table 1). The intensity of stained tumor cells was graded as either high or low. When setting the EV value, the immunohistochemical staining value of each EV marker was characterized as low with one point and as high with two points, and the sum of the scores for the five values was classified as low with six points and as high with more than seven points. Tumor-infiltrating immune cells were used as the internal control for each marker. Tumor cells that stained stronger than tumor-infiltrating immune cells were classified as having high expression, and other tumor cells were classified as having low expression. If tumor cells showed a heterogeneous pattern of expression in the same TMA core, the representative value was decided according to the majority of the tumor cells. All the samples were individually reviewed by two pathologists to confirm the reproducibility.

Statistical analysis

The relationships between EV marker expression and pathological or clinical data were evaluated by Pearson's chi-square test and Fisher's exact test. DFS and DSS were evaluated by the Kaplan-Meier method and a Cox proportional hazard regression model among each group. *P*-values less than 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS ver. 24.0 (IBM Corp., Armonk, NY, USA).

Results

Clinicopathological data of the patients

A total of 96 patients with lung SCC were enrolled in this study. The clinical and pathological parameters of the patients

Table 1 Relationship between each EV markers and clinicopathological characteristics

	CD9			CD63			LC3A/B			ANXA1			P62			EV		
Variables	Low	High	*P*-value	Low	High	*P*-value	Low	High	*P*-value	Low	High	*P*-value	Low	High	*P*-value	Low	High	*P*-value
Age (years)			0.231			0.517			**0.031**			0.155			0.566			0.160
<65	30	4		31	2		30	4		31	3		15	18		27	6	
≥65	47	13		54	6		41	19		48	12		31	29		41	19	
Gender			0.556			1.000			1.000			1.000			1.000			1.000
Male	74	16		82	8		69	22		76	15		45	45		66	24	
Female	3	1		3	0		2	1		3	0		1	2		2	1	
Smoking			0.546			0.675			0.413			0.753			0.593			0.809
Non-smoker	21	3		23	1		20	4		21	3		13	11		18	6	
Smoker	56	14		62	7		51	19		58	12		33	36		50	19	
Surgery			1.000			0.343			0.551			**0.016**			0.450			0.387
Lobectomy	63	14		68	8		57	20		68	9		39	37		57	19	
More invasive**	14	3		17	0		14	3		11	6		7	10		11	6	
Pathologic differentiation			0.754			0.388			0.828			**0.018**			0.301			0.168
W/D, M/D	58	14		66	5		54	18		57	15		33	38		49	22	
P/D	19	3		19	3		17	5		22	0		13	9		19	3	
T stage			**0.048**			1.000			**0.008**			0.519			0.218			0.229
T1	18	8		23	2		14	11		20	5		15	10		16	9	
T2,3,4	59	9		62	6		57	12		59	10		31	37		52	16	
N stage			0.140			0.470			0.405			0.591			0.162			0.980
N0	44	13		50	6		44	12		48	8		31	25		41	15	
N1,2,3	33	4		35	2		27	11		31	7		15	22		27	10	

*Bilobectomy, sleeve lobectomy or pneumonectomy; EV, representative value of panel (value = CD9 + CD63 + LC3A/B + ANXA1 + P62); M/D, moderately-differentiated; *P*, *P*-value; P/D, poorly-differentiated; W/D, well-differentiated.

The number of valid specimen is variable from 93 to 94 due to loss of core.

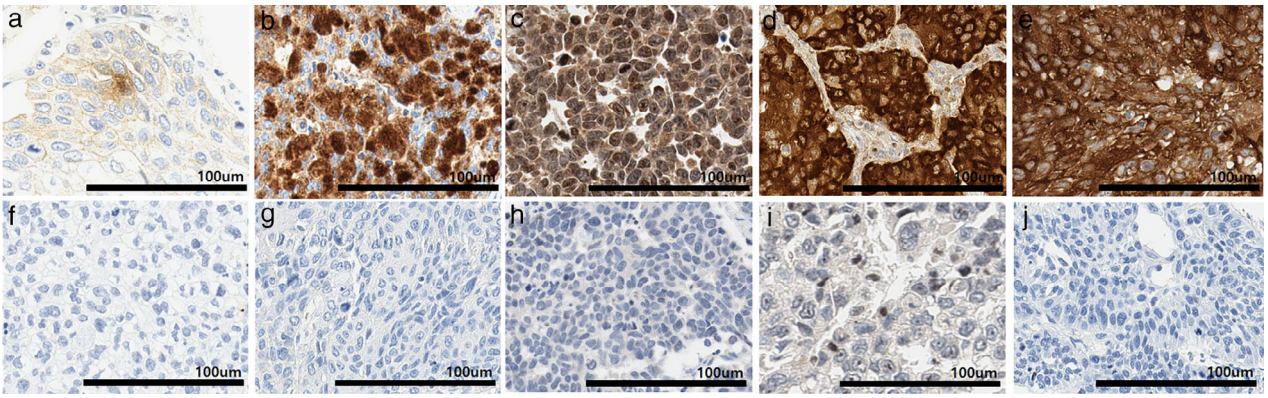

Figure 1 Expression of each extracellular vesicle marker in SSCs. (**a**) High membranous CD9 expression, (**b**) high nuclear expression for CD63, (**c**) high nuclear and cytoplasmic expression for LC3A/B and (**d**) P62, and (**e**) high cytoplasmic expression for ANXA1 was seen in tumor cells. In contrast, SCC tumor cells with (**f**) low CD9 expression, (**g**) low 63 expression, (**h**) low LC3A/B expression, (**i**) low P62 expression, and (**j**) low ANXA1 expression showed lower staining intensity than intratumoral immune cells.

were analyzed for their relationship with each EV marker, and the results are summarized in Table 1. The mean age of the patients was 65.68 years. Among them, 92 (95.8%) were males, and 72 (75.0%) had a smoking history. The pathological differentiation of tumors was as follows: 15 (15.6%) specimens were well-differentiated, 58 (60.4%) were moderately-differentiated, and 23 (24.0%) were poorly-differentiated. Of all the patients, 79 patients (82.3%) underwent lobectomy, while the remaining 17 patients (17.7%) underwent more invasive procedures, including bilobectomy, sleeve lobectomy, and pneumonectomy. In terms of TNM stage, 36 (37.6%) samples were stage I, 45 (46.9%) were stage II, 14 (14.6%) were stage III, and one (1.0%) was stage IV. The distribution of pathological T stages was as follows: T1mi, 0 (0%); T1a, 0 (0%); T1b, 12 (12.5%); T1c, 15 (15.6%); T2a, 33 (34.4%); T2b, 12 (12.5%); T3, 17 (17.7%); and T4, 7 (7.3%). The distribution of N stages was as follows: N0, 58 (60.4%); N1, 35 (36.5%); N2, three (3.1%); and N3, 0 (0%). The distribution of M stages was as follows: M0, 95 (99.0%); M1a, one (1.0%); M1b, 0 (0%); and M1c, 0 (0%).

Relationship between expression of each EV marker and clinicopathological data.

The relationships between the expression of each EV marker and patient characteristics are shown in Table 1. CD9 and LC3A/B expression was significantly related to pathological T stage (*P*-value = 0.048 and 0.008, respectively). In addition, LC3A/B expression was also significantly related to the patient's age (*P*-value = 0.031). Meanwhile, ANXA1 expression, a novel microvesicle marker, showed a statistically significant association with the operating method and pathological differentiation of SCC (*P*-value = 0.016 and 0.018, respectively). EV expression, which was a combination of the representative values of the immunohistochemical staining panel (value = CD9 + CD63 + LC3A/B + ANXA1 + P62), did not show any statistical relevance to any of the variables.

Staining patterns of each EV marker and their statistical significance

Samples with high CD9 expression (Fig 1a) showed weaker but diffuse membranous staining patterns than those with low CD9 expression (Fig 1f). Although the intensity of high CD9 expression was very weak, it was stronger than

Table 2 Statistical significance in each EV marker

P-value	CD9	CD63	LC3A/B	ANXA1	P62	EV
CD9		**0.027**	0.072	**0.001**	0.286	**<0.001**
CD63	**0.027**		0.948	**0.001**	0.124	**<0.001**
LC3A/B	0.072	0.948		0.588	0.152	**0.008**
ANXA1	**0.001**	**0.001**	0.588		0.998	**<0.001**
P62	0.286	0.124	0.152	0.998		**0.018**
EV	**<0.001**	**<0.001**	**0.008**	**<0.001**	**0.018**	

Note: *P*-values less than 0.05 were considered as significant and checked in bold. EV, representative value of panel (value = CD9 + CD63 + LC3A/B + ANXA1 + P62).

Table 3 Cox proportional hazards regression model of DFS and DSS for SCC patients (n = 96)

	Univariate analysis				Multivariate analysis			
	DFS		DSS		DFS		DSS	
Variables	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years) (<65 vs. ≥65)	1.308 (0.811–2.110)	0.270	1.230 (0.738–2.051)	0.427				
Gender (male vs. female)	0.519 (0.238–1.132)	0.099	0.316 (0.115–0.871)	**0.026**			0.355 (0.048–2.630)	0.311
Smoking (non-smoker vs. smoker)	0.844 (0.521–1.368)	0.492	0.983 (0.581–1.664)	0.950				
Surgery (lobectomy vs. more invasive**)	1.594 (0.854–2.973)	0.143	1.494 (0.759–2.944)	0.246				
Pathologic differentiation (W/D, M/D vs. P/D)	2.142 (1.201–3.821)	**0.010**	2.089 (1.130–3.861)	**0.019**	2.031 (1.088–3.794)	**0.026**	2.088 (1.117–3.902)	**0.021**
TNM stage (I, II vs. III, IV)	2.171 (1.270–3.711)	**0.005**	1.863 (1.026–3.385)	**0.041**	1.981 (1.008–3.892)	**0.047**	1.784 (0.872–3.650)	0.113
CD9 (low vs. high)	0.867 (0.481–1.560)	0.633	0.778 (0.404–1.496)	0.451				
CD63 (low vs. high)	0.515 (0.276–0.960)	**0.037**	0.606 (0.315–1.165)	0.133	0.981 (0.332–2.900)	0.972		
LC3A/B (low vs. high)	0.594 (0.313–1.130)	0.113	0.734 (0.382–1.411)	0.353				
ANXA1 (low vs. high)	0.725 (0.398–1.323)	0.295	0.821 (0.427–1.578)	0.555				
P62 (low vs. high)	0.995 (0.624–1.585)	0.983	1.278 (0.773–2.113)	0.338				
EV (low vs. high)	0.464 (0.268–0.801)	**0.006**	0.597 (0.337–1.059)	0.078	0.934 (0.459–1.901)	0.851		

*Bilobectomy, sleeve lobectomy or pneumonectomy; CI, confidence interval; DFS, disease-free survival; DSS, disease-specific survival; EV, representative value of panel (value = CD9 + CD63 + LC3A/B + ANXA1 + P62); HR, hazard ratio; M/D, moderately-differentiated; P/D, poorly-differentiated; W/D, well-differentiated. Note: *P*-values less than 0.05 were considered as significant and checked in bold.

that of immune cells. Samples with high CD63 expression (Fig 1b) demonstrated stronger patchy positive nuclear intensity than those with low CD63 expression (Fig 1g). Both the samples with high LC3 expression (Fig 1c) and the samples with high P62 expression (Fig 1d) showed strong and diffuse nuclear and cytoplasmic expression. Samples with low P62 expression (Fig. 1i) showed very weakly positive cytoplasmic staining patterns, and it was much weaker than that in the immune cells. High ANXA1 samples (Fig 1e) showed strong and diffuse cytoplasmic expression patterns compared with ANXA1-negative samples (Fig 1j). Table 2. demonstrates the *P*-values of chi-square tests between each EV marker on each TMA core. Among the five markers described above, CD9 and CD63 showed statistical significance (*P*-value = 0.027). In addition, ANXA1, a novel microvesicle marker and CD9 (*P*-value = 0.001) and CD63 (*P*-value = 0.001) showed statistical significance.

EV expression and survival analysis

The mean follow-up time of the patients in this study was 113 months. The median DSS time was 39 months. Of all the patients, 54.2% (n = 52) had disease recurrence, and 46.9% (n = 45) died due to lung SCC. The univariate Cox proportional hazards regression analysis of DFS and DSS showed that patients with SCC with low CD63 expression and patients with SCC low EV expression had unfavorable DFS rates (*P*-value = 0.037 and 0.006, respectively). To confirm CD63 and EV expression as independent prognostic factors, multivariate analysis was performed. However, SCC samples with low CD63 and SCC samples with low EV expression failed to show a statistically significant relationship with DFS in the multivariate analysis (Table 3). Meanwhile, the Kaplan-Meier survival curve confirmed that low EV expression had a statistically significant relationship with unfavorable DFS (*P*-value = 0.004) (Fig 2a) and a tendency to have a relationship with DSS (*P*-value = 0.072) (Fig 2b). There were no significant differences in DFS or DSS between the low and high expression groups for CD9, LC3A/B, ANXA1, and P62.

Discussion

For decades, exosomes have been known as key molecules for cell-to-cell communication to transport microRNA, mRNA, dsDNA, protein, and lipids to affect recipient cells.[8–16] Recently, however, Jeppesen *et al.* challenged how exosomes are classified and reclassified them based on their size and markers; classical exosomes (40–150 nm) and arrestin-domain-containing protein 1-mediated microvesicles (ARMMs) (~40–100 nm) are small EVs, classical microvesicles (~150–1000 nm) and apoptotic bodies (1–5 μm) are

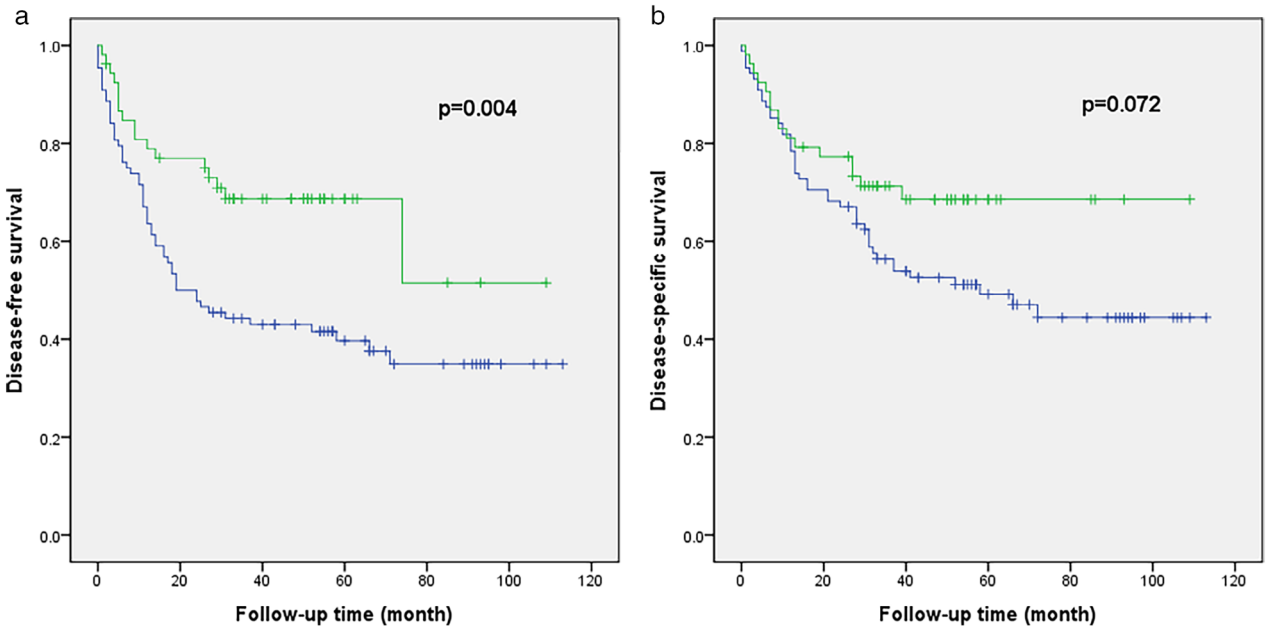

Figure 2 Survival analysis using the Kaplan-Meier method based on extracellular vesicle (EV) marker expression in samples of SCC of the lung. The low EV marker expression group showed significantly lower disease-free survival than the high EV marker expression group. low, high, low-censored, high-censored (**a**) and a tendency for decreased disease-specific survival (**b**), low, high, low-censored, high-censored.

large EVs, and nonvesicular fractions (NFs) are non-extracellular vesicles.[6] They suggested that extracellular vesicles have a different composition of RNA, DNA, and protein according to their size.[6] Intracellularly, autophagosomes usually fuse with a lysosome to degrade internal cargo, creating autophagolysosomes. However, sometimes, MVEs may fuse with autophagosomes to make amphisomes. While CD63 and CD9 are well-known exosomal markers (they both are specific for isolated exosomes and multivesicular endosomes within the cell), P62 and LC3 are autophagosomal markers. Naturally, amphisomes may show colocalized expression of CD63, CD9, P62, or LC3A/B, and amphisomes eventually fuse with the cell plasma membrane for exocytosis of NFs, which contain nonvesicular extracellular matter of histones and dsDNA. Exosomes have been known to have abundant RNA cargos, including miRNAs, which are sorted and packaged into the exosome with the help of Y-box protein 1.[17, 18] When extracellular RNA was extracted and RNA sequencing was employed, however, most microRNAs were more associated with the extracellular NV fraction than with parental cells or small EVs.[6] Additionally, many microRNAs showed different distributions between small EVs and extracellular NV fractions.[6] Since microRNA is an important molecule that works mostly as a tumor suppressor gene in tumor cells, and for the reasons mentioned above, we believe that treatments for lung SCC should not be limited to exosomes alone.

The EV markers we used in this study are markers for the precursor materials of EVs within the cells: CD63 and CD9 for MVEs, LC3A/B, and P62 for amphisomes, and ANXA1 for microvesicles. Hypothetically, if there are many precursor substances that are positive for EV markers intracellularly, there would be many EVs released out of the cell, and tumor exosomes released from tumor cells in such a method would affect tumor processes such as tumor cell metastasis. In this study, classical exosome markers (CD9 and CD63) and microvesicle marker (ANXA1) showed statistical significance in lung SCC cells in vivo. While Dennis *et al.* tested EV markers in cell line experiments, we assessed EV markers in tissue specimens of lung SCC. To the best of our knowledge, the EV expression, a combination of the representative values of the immunohistochemical staining panel (value = CD9 + CD63 + LC3A/B + ANXA1 + P62) have never been tested in vivo. In the survival analysis, SCC samples with low CD63 and EV expression showed poor DFS. Meanwhile, the Kaplan-Meier survival curve confirmed that samples with low EV expression showed a statistically significant relationship with unfavorable DFS (p-value = 0.004). It is possible that the lower the amount of precursors of EVs is, the lower the amount of secreted exosomes or NFs. Being trapped in tumor cells, MVEs or amphisomes may be degraded with their cargos, most importantly, microRNAs.[19] Eventually, this will break the balance in favor of metastasis for the tumor cells. A limitation of this study is that we did not detect any other factors that might have influenced the exocytosis of MVEs, for example, secretory Rabs, which lead to accelerated exosome

secretion.[19, 20] However, given our previous data, we assumed that multiple factors must associate with EVs, which might affect the prognosis of patients with SCC of the lung. In addition, there were no significant differences in DFS or DSS between the low and high expression groups for CD9, LC3A/B, ANXA1, and P62. We believe that EV expression, which considered the effects of each EV marker, better reflected patient survival than each separate EV marker. Considering all of the different extracellular vesicles rather than just exosomes[10, 12, 13] may be necessary.

In conclusion, the combined expression of CD9, CD63, LC3A/B, ANXA1, and P62 as a marker for extracellular vesicles is effective in predicting the prognosis of patients with SCC of the lung. A broader approach using different extracellular vesicles rather than conventional exosome-dependent methods is required when diagnosing and treating lung cancer patients. The prognostic role of EV expression and its association with DFS in SCC of the lung were elucidated in this study.

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Disclosure

No potential conflicts of interest relevant to this article were reported.

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