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Basic Research

Prognostic Role of Circulating Tumor Cells in the Pulmonary Vein, Peripheral Blood, and Bone Marrow in Resectable Non-Small Cell Lung Cancer

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Background: Studies of the prognostic role of circulating tumor cells (CTCs) in early-stage non-small cell lung cancer (NSCLC) are still limited. This study investigated the prognostic power of CTCs from the pulmonary vein (PV), peripheral blood (PB), and bone marrow (BM) for postoperative recurrence in patients who underwent curative resection for NSCLC.

Methods: Forty patients who underwent curative resection for NSCLC were enrolled. Before resection, 10-mL samples were obtained of PB from the radial artery, blood from the PV of the lobe containing the tumor, and BM aspirates from the rib. A microfabricated filter was used for CTC enrichment, and immunofluorescence staining was used to identify CTCs.

Results: The pathologic stage was stage I in 8 patients (20%), II in 15 (38%), III in 14 (35%), and IV in 3 (8%). The median number of PB-, PV-, and BM-CTCs was 4, 4, and 5, respectively. A time-dependent receiver operating characteristic curve analysis showed that PB-CTCs had excellent predictive value for recurrence-free survival (RFS), with the highest area under the curve at each time point (first, second, and third quartiles of RFS). In a multivariate Cox proportional hazard regression model, PB-CTCs were an independent risk factor for recurrence (hazard ratio, 10.580; 95% confidence interval, 1.637–68.388; p<0.013).

Conclusion: The presence of ≥4 PB-CTCs was an independent poor prognostic factor for RFS, and PV-CTCs and PB-CTCs had a positive linear correlation in patients with recurrence.

Keywords: Circulating tumor cells, Non-small-cell lung carcinoma, Pulmonary veins, Bone marrow, Peripheral blood

Introduction

Lung cancer is the leading cause of cancer death for both men and women worldwide. The 5-year survival rate is estimated to range from 71% to 83% in patients with pathologic stage I disease and from 23% to 36% in patients with stage III non-small cell lung cancer (NSCLC) [1]. The reason for the poorer prognosis in advanced disease is postoperative recurrence; 80% of cases of recurrence occur within the first 2 years, and most recurrences are distant metastases [2]. The mechanism of distant metastasis is known to be nodal spread or spread through the bloodstream.

Circulating tumor cells (CTCs) disseminate from the primary tumor through the circulatory system, and at least some CTCs are ultimately capable of forming distant metastases [3]. However, it is clear that even in advanced lung cancer, CTCs exist in extreme rarity in blood, and there are significant technical challenges in their isolation. However, technical advances have enabled the identification of CTCs in peripheral blood (PB), and some studies showed that CTCs can be harvested from numerous cancer subtypes, such as breast, colon, lung, prostate, and urothelial cancers [4-8].

Blood from the pulmonary vein (PV) responsible for

drainage from the area of the primary tumor is known to be better than blood from the PB for research on the identification of CTCs because it contains a tremendous amount of CTCs [9-12]. Bone marrow (BM) has traditionally been the primary compartment in which the prognostic value of the detection of CTCs has been investigated, as it is a common homing organ for tumor cells of epithelial origin [13,14]. Most studies on CTCs to date have been performed in advanced NSCLC, and studies addressing the prognostic role of CTCs in early-stage NSCLC are still limited. One reason for the limited number of studies in early-stage NSCLC is that isolation of CTCs is more challenging due to their rarity.

This study was to evaluate the prognostic effect of CTCs from PB, PV, and BM on postoperative recurrence in patients who underwent curative resection for NSCLC.

Methods

Patients

Patients enrolled in this study underwent curative resection at Seoul National University Bundang Hospital (SNUBH) from January 2015 to August 2016 and were pathologically diagnosed with primary NSCLC. All patients underwent complete anatomical resection with mediastinal lymph node dissection. The tumors were staged pathologically according to the eighth edition of the tumor-node-metastasis (TNM) classification. This study was approved by the Institutional Review Board of SNUBH (IRB no., B-1410-270-001) and informed consent was obtained from all patients. The data analyzed in this study included the patient's history, physical examination, chest radiographs, and tumor markers. A contrast-enhanced chest computed tomography scan was taken every 6 months for the first 3 years and every 8 months thereafter. The final diagnosis of recurrence was confirmed by the histopathologic examination of samples obtained from surgery or biopsy. If it was impossible to diagnose recurrence histopathologically, recurrent malignancy was no longer suspected based on a clinical and radiologic follow-up period of at least 12 months with no evidence of active malignancy.

Sample collection

After induction for general anesthesia, a total of 10 mL of PB was collected immediately prior to surgery from radial artery. The operation was performed by video-assisted thoracic surgery (VATS) or open thoracotomy due to oncologic reasons or technical difficulty. BM was collected from patients before lung resection, and a total of 10 mL of BM was aspirated from the fifth or sixth rib. After exposure of the targeted rib, using a bone drill, we made a hole in the rib, inserted the spinal needle in the hole, and used a 20-mL syringe, to aspirate 5 mL of BM. If the amount was insufficient, BM was aspirated from another rib. After entering the thoracic cavity, we first exposed the targeted PV (draining the lobe that was bearing the tumor) and isolated the PV first using minimal manipulation of the lung. Up to 10 mL of PV blood was removed using a 21G needle attached to a 10-mL syringe immediately before dividing the PV. All specimens were collected in specialized tubes and then submitted for an immediate analysis.

Technique of enrichment and identification of circulating tumor cells

The technique of CTC enrichment and detection was described in detail previously [15]. Briefly, the principle of isolating CTCs by filtration is based on the fact that most CTCs are larger than most blood cells. Most cancer cells measure more than 15 μm in size, whereas most PB leukocytes measure from 8 to 11 μm. Samples were processed using an epithelial cell adhesion molecule (EpCAM)-based microfluidic chip. CK, DAPI, and CD45 labeling was used to score for CTCs and eliminate contaminating white blood cells. CK is positive in many CTCs, DAPI is positive in nucleated cells, eliminating red blood cells, and CD45 is positive in white blood cells, allowing for negative selection.

Statistical analysis

Continuous were expressed as mean±standard deviation or median with the range between the first and third quartiles, and categorical data were expressed as proportions. The baseline characteristics of patients were compared between 2 groups using the Wilcoxon rank sum for continuous variables and the Fisher exact test for categorical variables. To measure the linear relationship between variables, simple linear regression analysis was conducted. To evaluate the prediction efficacy of several variables for recurrence, the time-dependent receiver operating characteristic (ROC) curve and area under time-dependent ROC curve (AUC) were estimated at each time point (first, second, and third quartiles of recurrence-free survival [RFS]). Tests to compare the AUCs of 2 rival variables were also conducted

to compare the prediction efficacy. RFS was defined as the duration between the day of surgery and the day that the recurrence of lung cancer was detected. Overall survival (OS) was defined as the duration from the day of surgery until the day of death. The RFS and OS rates were estimated using the Kaplan-Meier method. A Cox regression model was used to analyze the risk of lung cancer recurrence. To reduce the influence of confounding factors on the risk, adjustment was done using multiple variables. Due to complete and quasi-complete separation (empty occurrence categories), the firth penalized maximum likelihood was applied [16]. A p-value of <0.05 was considered to indicate statistical significance. Statistical analyses were performed using R statistical software ver. 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria) and IBM SPSS Statistics for Windows ver. 25.0 (IBM Corp., Armonk, NY, USA). A 2-sided p-value <0.05 was considered statistically significant.

Results

Patient demographics

The baseline characteristics of the study cohort are shown in Table 1. Of all 40 patients, the mean age was 65.3 years, 25 patients (63%) were male, and 25 patients (63%) were ever smokers. The mean maximum standardized uptake value (SUVmax) of the primary tumor was 11.3. Lobectomy was performed in 35 patients (88%), bilobectomy in 3 (8%), and pneumonectomy in 2 (5%). The procedures were performed by VATS in 27 patients (68%). The proportions of patients with adenocarcinoma, squamous cell carcinoma, and other types of NSCLC were 27 (68%), 10 (25%), and 3 (8%), respectively. Their pathologic TNM stages were as follows: stage I in 8 (20%), stage II in 15 (38%), stage III in 14 (35%), and stage IV in 3 (8%).

Relationships between pulmonary vein-, peripheral blood-, and bone marrow-circulating tumor cells and clinicopathologic factors

The median numbers of PB-, PV-, and BM-CTCs were 3 (1–6), 3 (1–6), and 5 (1–10), respectively. Table 1 shows clinicopathologic features according to the number of PV-, PB-, and BM-CTCs. In each column, patients were divided into 2 groups—the high CTCs group (>median number) and the low CTCs group (≤median number)—and their clinicopathologic features were compared. The proportions of patients in the high PV-, PB-, and BM-CTCs groups were 19 (48%), 19 (48%), and 18 (45%), respectively. Statistically significant correlations were observed between PV-CTCs and pathologic N stage, and PB-CTCs and adenocarcinoma. Marginal significance was observed between PV-CTCs or PB-CTCs and preoperative distant metastasis. Non-significant relationships were found between CTCs and visceral pleural invasion (VPI), lymphatic invasion (LI) or vascular invasion (VI), or spread through air spaces (STAS).

Pulmonary vein-, peripheral blood-, and bone marrow-circulating tumor cells and pathologic TNM stage

The scatterplots of PV-, PB-, and BM-CTCs according to the pathologic tumor-node-metastasis stage (pTNM) are shown in Fig. 1. There was no correlation between CTCs and pTNM, except PB-CTCs and pM. In the linear regression analysis, PB-CTCs and pM showed an R^2 of 0.230 with a p-value of 0.002. Further details on PB–CTCs and pM are shown in Supplementary Fig. 1. In all 3 patients with pM1, the number of PB-CTCs was 8 or more. Among patients with pM0, all patients with ≥8 PB-CTCs (n=5) had postoperative recurrence. In the ROC analysis, the AUC of PB-CTC for pM1 was 0.923 (p<0.001), and the cut-off value that maximized the Youden index was 7.

Correlations between pulmonary vein-, peripheral blood-, and bone marrow-circulating tumor cells and postoperative recurrence

Scatterplots of PV-, PB-, and BM-CTCs according to recurrence status are presented in Fig. 2A. When the number of CTCs was compared between patients with recurrence and without recurrence, the numbers of PV- and PB-CTCs were significantly higher in patients with recurrence (Fig. 2B). The results of the time-dependent ROC analysis of PV-, PB-, and BM-CTCs for recurrence are presented in Fig. 2C. At each time point (first, second, and third quartiles of RFS), PB-CTCs showed excellent predictive values and the greatest AUC when compared to PV- and BM-CTCs. The cut-off value of PB-CTCs for recurrence determined using the maximum Youden index was 4 when the time point was 55 months. The cut-off of 4 PB-CTCs discriminated RFS well in Kaplan-Meier curves (log-rank p=0.001) (Fig. 2D).

Table 1. Baseline characteristics of the study cohort and correlations between the number of CTCs and clinicopathological features

Characteristic	Study cohort (n=40)	PV-CTC			PB-CTC			BM-CTC		
		0–3 (n=21)	≥4 (n=19)	p-value	0–3 (n=21)	≥4 (n=19)	p-value	0–5 (n=22)	≥6 (n=18)	p-value
Circulating tumor cells										
PV	3 (1–6)	1 (0–2)	7 (5–9)	<0.001	2 (0–4)	4 (2–8)	0.081	2 (1–5)	5 (2–8)	0.069
PB	3 (1–6)	2 (0–4)	6 (3–10)	0.004	1 (0–3)	7 (6–11)	<0.001	3 (1–6)	6 (3–10)	0.057
BM	5 (1–10)	5 (0–8)	7 (2–11)	0.242	5 (0–12)	6 (2–10)	0.950	2 (0–3)	11 (9–14)	<0.001
Age (yr)	65.3±8.9	66.8±9.6	63.7±7.9	0.267	67.1±8.9	63.4±8.7	0.200	65.5±9.7	65.1±8.0	
Sex, male	25 (62.5)	14 (66.7)	11 (57.6)	0.572	14 (66.7)	11 (57.9)	0.572	8 (36.4)	7 (38.9)	0.871
Smoking, ever	25 (62.5)	14 (66.7)	11 (57.9)	0.572	14 (66.7)	11 (57.9)	0.572	14 (63.6)	11 (61.1)	0.871
SUVmax	11.3±7.5	12.57±9.03	11.20±7.27	0.627	12.24±9.05	11.46±7.16	0.777	13.84±9.22	9.23±5.49	0.075
CEA	2.3 (1.5–4.7)	9.81 (29.77)	58.41 (222.86)	0.371	49.73 (206.57)	11.82 (32.13)	0.447	9.13 (29.75)	59.19 (222.68)	0.357
Cyfra 21-1	2.1 (1.5–2.9)	8.46 (23.37)	5.58 (9.50)	0.619	5.37 (9.73)	9.22 (24.95)	0.555	9.68 (23.24)	4.15 (9.19)	0.337
NSE	21.9 (16.9–27.7)	24.21 (10.12)	23.03 (9.57)	0.715	23.98 (8.90)	23.31 (11.00)	0.842	24.23 (11.28)	23.00 (7.80)	0.693
cT				0.931			0.698			0.897
1	6 (15.0)	1 (4.8)	3 (15.8)		2 (9.5)	2 (10.5)		4 (18.2)	0	
2	24 (60.0)	17 (81.0)	10 (52.6)		15 (71.4)	12 (63.2)		13 (59.1)	14 (77.8)	
3	10 (25.0)	3 (14.3)	6 (31.6)		4 (19.0)	5 (26.3)		5 (22.7)	4 (22.2)	
cN				0.708			0.886			0.578
0	19 (47.5)	16 (76.2)	14 (73.7)		16 (76.2)	14 (73.7)		17 (77.3)	13 (72.2)	
1	9 (22.5)	4 (19.0)	2 (10.5)		3 (14.3)	3 (15.8)		4 (18.2)	2 (11.1)	
2	12 (30.0)	1 (4.8)	2 (10.5)		1 (4.8)	2 (10.5)		1 (4.5)	2 (11.1)	
cM				0.293			0.293			0.269
0	39 (97.5)	0	1 (5.3)		1 (4.8)	0		0	1 (5.6)	
1	1 (2.5)	21 (100.0)	18 (94.7)		21 (100.0)	18 (94.7)		22 (100.0)	17 (94.4)	
Tumor location				0.247			0.630			0.125
RUL	14 (35.0)	9 (42.9)	5 (26.3)		7 (33.3)	7 (36.8)		10 (45.5)	4 (22.2)	
RML	2 (5.0)	2 (9.5)	0		0	2 (10.5)		0	2 (11.1)	
RLL	8 (20.0)	3 (14.3)	5 (26.3)		4 (19)	4 (21.1)		5 (22.7)	3 (16.7)	
LUL	9 (22.5)	4 (19)	5 (26.3)		7 (33.3)	2 (10.5)		5 (22.7)	4 (22.2)	
LLL	7 (17.5)	3 (14.3)	4 (21.1)		3 (14.3)	4 (21.1)		2 (9.1)	5 (27.8)	
Approach				0.948			0.948			0.130
VATS	27 (67.5)	18 (85.7)	16 (84.2)		18 (85.7)	16 (84.2)		17 (77.3)	17 (94.4)	
Thoracotomy	13 (32.5)	3 (14.3)	3 (15.8)		3 (14.3)	3 (15.8)		5 (22.7)	1 (5.6)	
Extent of resection				0.654			0.179			0.831
Lobectomy	35 (87.5)	18 (85.7)	17 (89.5)		17 (81.0)	18 (94.7)		19 (86.4)	16 (88.9)	
Bilobectomy	3 (7.5)	1 (4.8)	2 (10.5)		2 (9.5)	1 (5.3)		2 (9.10)	1 (5.6)	
Pneumonectomy	2 (5.0)	2 (9.5)	0		2 (9.5)	0		1 (4.5)	1 (5.6)	

(Continued on next page)

Table 1. Continued

Characteristic	Study cohort (n=40)	PV-CTC			PB-CTC			BM-CTC		
		0–3 (n=21)	≥4 (n=19)	p-value	0–3 (n=21)	≥4 (n=19)	p-value	0–5 (n=22)	≥6 (n=18)	p-value
Cell type				0.681			0.025			0.228
Adenocarcinoma	27 (67.5)	14 (66.7)	13 (68.4)		11 (47.4)	16 (84.2)		13 (59.1)	14 (77.8)	
Squamous cell carcinoma	10 (25.0)	4 (19.0)	6 (31.6)		7 (33.3)	3 (15.8)		7 (31.8)	3 (16.7)	
Others	3 (7.5)	3 (14.3)	0		3 (14.3)	0		2 (9.1)	1 (5.6)	
pT				0.660			0.607			0.988
1	6 (15.0)	3 (14.3)	3 (15.8)		4 (19.0)	2 (10.5)		5 (22.7)	1 (5.6)	
2	20 (50.0)	10 (47.6)	10 (52.6)		8 (38.1)	12 (63.2)		8 (36.4)	12 (66.7)	
3	11 (27.5)	6 (28.6)	5 (26.3)		7 (33.3)	4 (21.1)		7 (31.8)	4 (22.2)	
4	3 (7.5)	2 (9.5)	1 (5.3)		2 (9.5)	1 (5.3)		2 (9.1)	1 (5.6)	
pN				0.046			0.672			0.230
0	19 (47.5)	13 (61.9)	6 (31.6)		11 (52.4)	8 (42.1)		12 (54.5)	7 (38.9)	
1	9 (22.5)	4 (19.0)	5 (26.3)		4 (19.0)	5 (26.3)		5 (22.7)	4 (22.2)	
2	11 (27.5)	4 (19.0)	7 (36.8)		5 (23.8)	6 (31.6)		5 (22.7)	6 (33.3)	
3	1 (2.5)	0	1 (2.5)		1 (4.8)	0		0	1 (5.6)	
pM				0.062			0.062			0.439
0	37 (92.5)	21 (100.0)	16 (84.2)		21 (100.0)	16 (84.2)		21 (95.5)	16 (88.9)	
1	3 (7.5)	0	3 (15.8)		0	3 (15.8)		1 (4.5)	2 (11.1)	
p-stage				0.149			0.539			0.615
I	8 (20.0)	5 (23.8)	3 (15.8)		5 (23.8)	3 (15.8)		5 (22.7)	3 (16.7)	
II	15 (37.5)	9 (42.9)	6 (31.6)		7 (33.3)	8 (42.1)		8 (36.4)	7 (38.9)	
III	14 (35.0)	7 (33.3)	7 (36.8)		9 (42.9)	5 (26.3)		8 (36.4)	6 (33.3)	
IV	3 (7.5)	0	3 (15.8)		0	3 (15.8)		1 (4.5)	2 (11.1)	
Visceral pleural invasion	14 (35.0)	6 (28.6)	8 (42.1)	0.376	5 (23.8)	9 (47.4)	0.123	7 (31.8)	7 (38.9)	0.645
Vascular invasion	17 (42.5)	9 (42.9)	8 (42.1)	0.962	8 (38.1)	9 (47.4)	0.559	7 (31.58)	10 (55.6)	0.136
Lymphatic invasion	24 (60.0)	12 (57.1)	12 (63.2)	0.702	10 (47.6)	14 (73.7)	0.097	11 (50.0)	13 (72.2)	0.159
STAS	22 (55.0)	10 (47.6)	12 (63.2)	0.330	9 (42.9)	13 (68.4)	0.109	10 (45.5)	12 (66.7)	0.262
Adjuvant therapy	23 (57.5)	9 (42.9)	14 (73.7)	0.052	9 (42.9)	14 (73.7)	0.052	12 (54.5)	11 (61.1)	0.680
Chemotherapy	23 (57.5)	9 (42.9)	14 (73.7)	0.052	9 (42.9)	14 (73.7)	0.052	12 (54.5)	11 (61.1)	0.680
Chemoradiotherapy	6 (15.0)	3 (14.3)	3 (15.8)	0.896	4 (19.0)	2 (10.5)	0.457	3 (13.6)	3 (16.7)	0.792
Follow-up period (mo)	49.9 (44.5–55.4)	46.4 (42.9–56.1)	52.9 (45.3–54.7)	0.414	45.5 (36.0–56.1)	45.5 (36.0–56.1)	0.592	49.2 (42.9–56.2)	47.7 (45.1–54.5)	0.972
Recurrence	15 (119.1)	5 (72.5)	10 (75.7)		2 (26.3)	13 (260.5)	<0.001	6 (81.5)	9 (172.3)	0.181

Values are presented as number (%), mean±standard deviation, median (1st to 3rd quartile), or event (incidence rate in 1,000 person-years).
CTC, circulatory tumor cell; PV, pulmonary vein; PB, peripheral blood; BM, bone marrow; SUVmax, maximum standardized uptake value; CEA, carcinoembryonic antigen; NSE, neuron-specific enolase; TNM, tumor-node-metastasis; c, clinical; RUL, right upper lobe; RML, right middle lobe; RLL, right lower lobe; LUL, left upper lobe; LLL, left lower lobe; VATS, video-assisted thoracic surgery; p, pathologic; p-stage, pathological stage; STAS, spread through air spaces.

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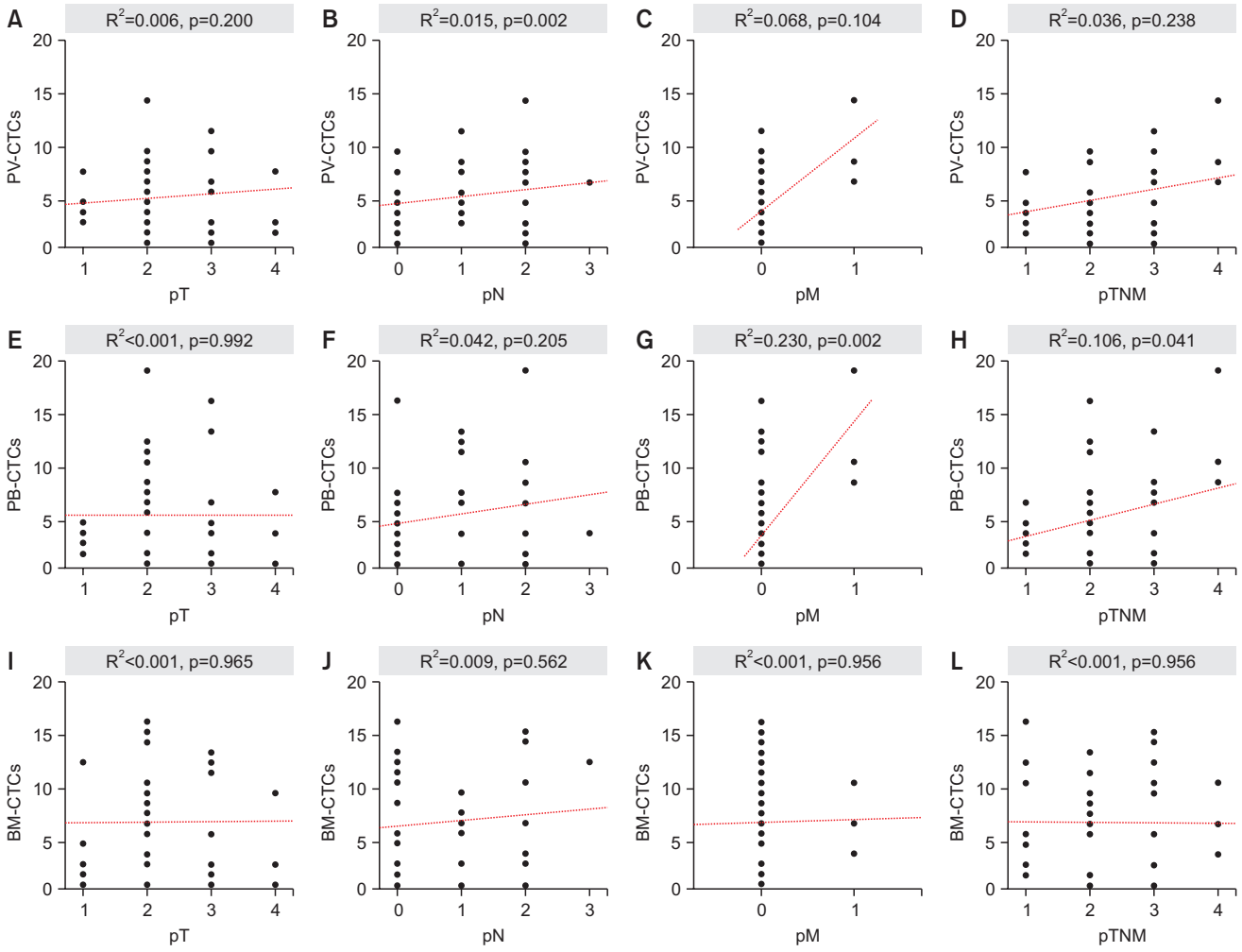

Fig. 1. Correlation between pathologic tumor-node-metastasis (TNM) stage and the number of (A–D) pulmonary vein (PV)-, (E–H) peripheral blood (PB)-, and (I–L) bone marrow (BM)-circulating tumor cells (CTCs).

Predictive power for postoperative recurrence

When the AUC of the ROC analysis for recurrence was compared between PB-CTCs and other well-known prognostic factors such as pTNM stage, SUVmax, and carcinoembryonic antigen (CEA), PB-CTCs showed a higher AUC than all other factors; its significance was marginal when compared to the pTNM stage, and it was significant when compared to SUVmax or CEA (Fig. 3). The univariate analysis is shown in Table 2. In the multivariate Cox proportional hazard regression model for recurrence, PB-CTCs were an independent risk factor for recurrence (hazard ratio, 38.952; 95% CI, 5.234–396.843; p<0.001) after adjustment for age, sex, extent of resection, completeness of resection, cell type, pathologic TNM stage, VPI, LI, VI, STAS, and adjuvant therapy (Table 3).

The interrelationships between PV-, PB-, and BM-CTCs are presented in Fig. 4. PV-CTCs and PB-CTCs showed a positive linear relationship (R^2=0.432, p=0.008) only when there was recurrence. In contrast, PV-CTCs and BM-CTCs showed a positive linear relationship (R^2=0.363, p=0.001) only when there was no recurrence.

Discussion

This study showed that PB-CTCs were an independently significant prognostic factor for RFS after curative resection for NSCLC. In this study, according to the results of time-dependent ROC analysis of 3 samples for recurrence prediction, PB-CTCs showed excellent predictive value, and a cut-off of 4 for PB-CTCs discriminated RFS well. At each time point (first, second, and third quartiles of RFS),

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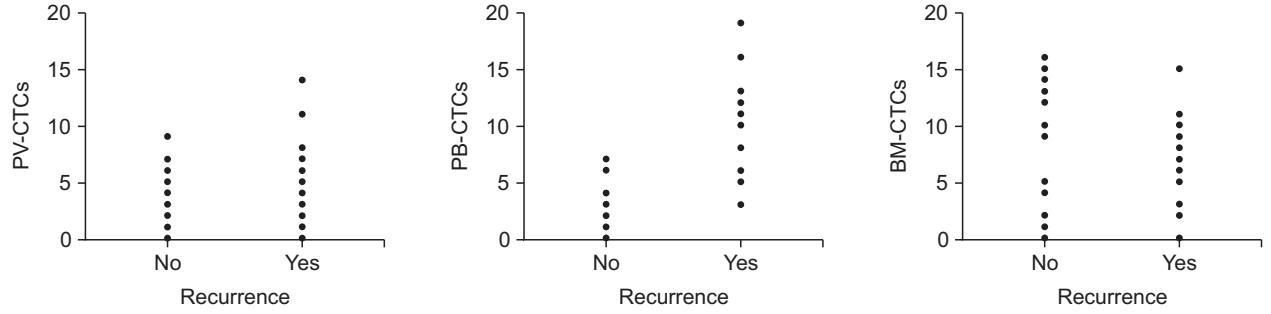
A



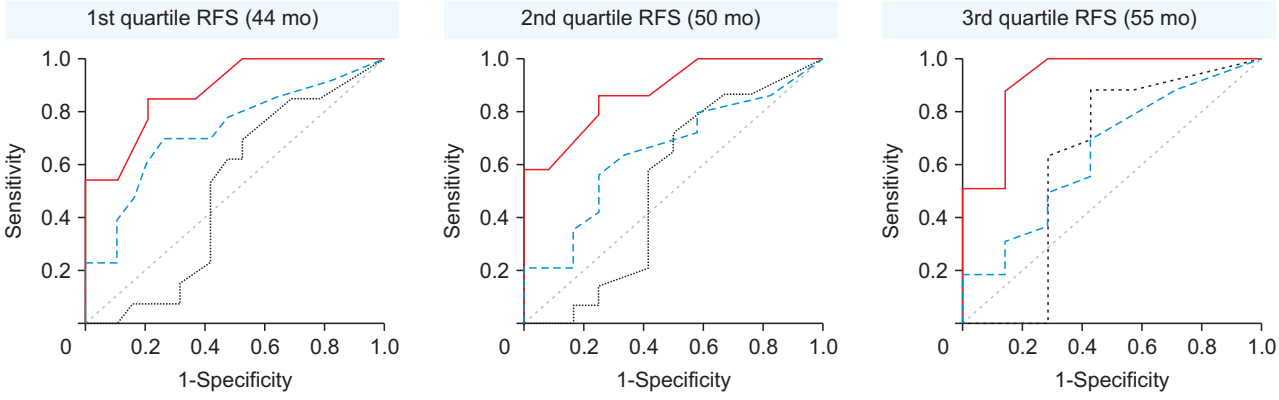
B

	PV-CTCs	PB-CTCs	BM-CTCs
No recurrence (n=25)	2 (1–4)	2 (1–3)	4 (0–12)
Recurrence (n=15)	6 (2–8)	8 (6–12)	6 (2–10)
p-value	0.013	<0.001	0.915

Values are presented as median (1st to 3rd quartile).

C

Time point (RFS)	AUC (95% CI)			Comparison of AUC (p-value)	
	PV-CTCs	PB-CTCs	BM-CTCs	PB vs. PV	PB vs. BM
1st quartile (44 mo)	72.5 (51.2–89.3)	87.9 (75.4–99.2)	50.2 (33.0–75.7)	0.110	0.013
2nd quartile (50 mo)	64.9 (43.7–86.0)	87.7 (75.4–100.0)	82.2 (28.1–76.3)	0.051	0.005
3rd quartile (55 mo)	64.2 (39.4–89.1)	92.1 (78.6–100.0)	62.3 (30.9–93.7)	0.034	<0.001



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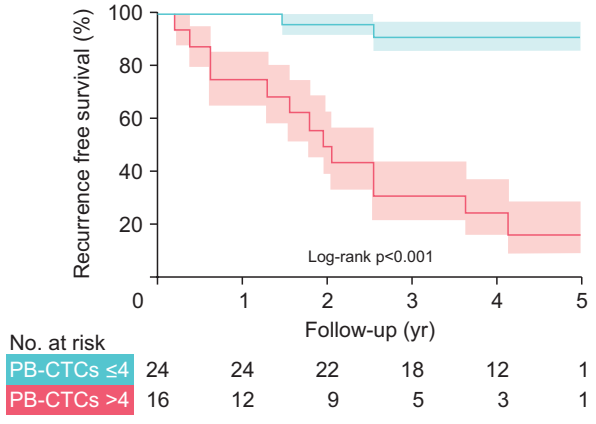

Fig. 2. 0 (A) Scatterplots of pulmonary vein (PV)-, peripheral blood (PB)-, and bone marrow (BM)-circulating tumor cells (CTCs) compared with recurrence status. (B) Comparison of PV-, PB-, and BM-CTCs according to recurrence status. (C) Time-dependent receiver operating characteristic (ROC) analysis of PV-, PB-, and BM-CTCs for recurrence and its comparisons. (D) Kaplan-Meier curves for recurrence-free survival (RFS) with 95% confidence intervals (CIs) stratified by the presence of ≥4 or <4 PB-circulating tumor cells (CTCs). AUC, area under the ROC curve.

PB-CTCs showed excellent predictive value and the highest AUC for the third quartile of RFS compared to PV- and BM-CTCs. A meta-analysis showed that CTCs were significantly correlated with overall survival and progression-free survival in lung cancer patients [17]. Although many studies have shown similar results, the first point of

Time point (RFS)	AUC (95% CI)				Comparison of AUC (p-value)		
	PB-CTCs	pTNM	SUVmax	CEA	PB-CTCs vs. pTNM	PB-CTCs vs. SUVmax	PB-CTCs vs. CEA
1st quartile (44 mo)	87.9 (75.4–99.2)	71.2 (56.5–90.2)	56.5 (29.6–74.0)	52.7 (32.4–76.3)	0.140	0.005	0.005
2nd quartile (50 mo)	87.7 (75.4–100.0)	77.4 (60.0–94.8)	57.6 (34.1–81.1)	54.5 (31.2–77.7)	0.315	0.026	0.026
3rd quartile (55 mo)	92.1 (78.6–100.0)	72.4 (50.3–94.4)	50.4 (23.9–76.9)	51.8 (24.8–78.7)	0.107	0.004	0.004

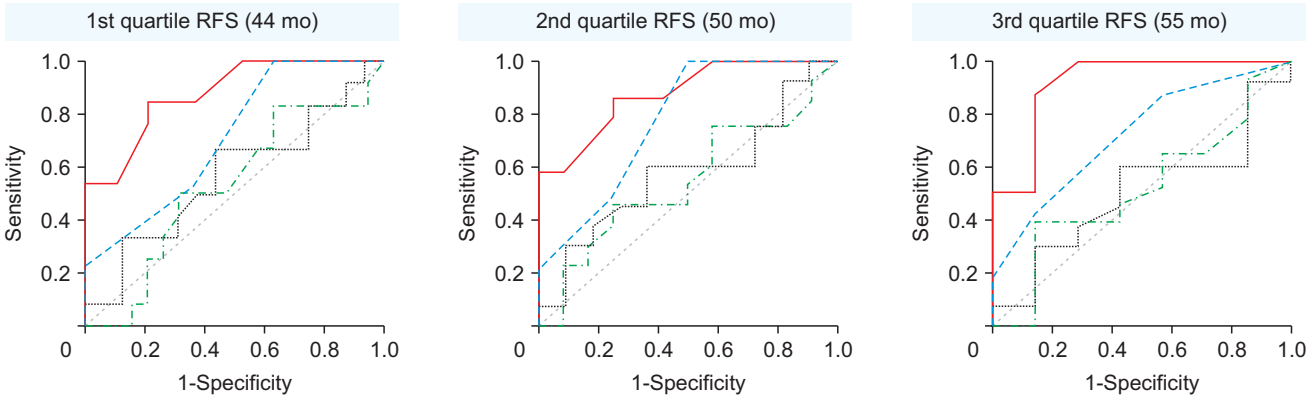

Fig. 3. Time-dependent receiver operating characteristic (ROC) analysis of peripheral blood (PB)-circulating tumor cells (CTCs), pathologic tumor-node-metastasis (pTNM), standardized uptake value (SUV), and carcinoembryonic antigen (CEA) for recurrence and its comparisons. AUC, area under the ROC curve; CI, confidence interval.

Table 2. Univariate analysis for recurrence

Variable	HR (95% CI)	p-value
Age	0.967 (0.913–1.025)	0.261
Sex, male (vs. female)	0.918 (0.333–2.526)	0.868
Smoking, ever (vs. never)	1.002 (0.356–2.816)	0.998
Carcinoembryonic antigen	0.988 (0.947–1.032)	0.596
Maximum standardized uptake value	1.023 (0.952–1.098)	0.539
Extent of resection, lobectomy (vs. bilobectomy or pneumonectomy)	1.000 (0.225–4.441)	1.000
Pathologic TNM		
Stage II (vs. I)	5.503 (0.668–45.354)	0.113
Stage III (vs. I)	3.975 (0.459–34.409)	0.210
Stage IV (vs. I)	20.353 (2.015–205.603)	0.011
Visceral pleural invasion, present (vs. absent)	2.180 (0.810–5.864)	0.123
Vascular invasion, present (vs. absent)	1.451 (0.544–3.8)	0.457
Lymphatic invasion, present (vs. absent)	2.557 (0.821–7.958)	0.105
Spread through air spaces, present (vs. absent)	2.936 (0.943–9.141)	0.063
Adjuvant therapy, yes (vs. no)	4.095 (1.164–14.407)	0.028
PB-CTCs >4 (vs. ≤4)	9.815 (2.225–43.896)	0.003

HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis; PB, peripheral blood; CTCs, circulating tumor cells.

note in our study is that it analyzed surgically resected lung cancer patients, and the second was that the analyzed samples were not only limited to PB, but also included samples from the PV and BM, unlike similar previous studies. Interestingly, in our patients with recurrence, PV-CTCs and PB-CTCs showed a positive linear relationship, meaning that a high number of PV-CTCs was maintained in the peripheral circulation. Previous data suggest that most CTCs are cleared from the circulation rapidly and only a very small fraction proliferate at a distant site [18,19]. Abundant immunocytes including T and B lymphocytes, and natural killer (NK) cells in the PB could kill the cancer cells that desquamate into the PB [20,21]. One study showed a close relationship between the decrease of peripheral immune surveillance and CTCs; this process was characterized by decreased ratios of NK cells, CD3+,

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Table 3. Multivariate firth penalized Cox hazard regression analysis for recurrence

Variable	HR (95% CI)	p-value
Age	0.859 (0.756–0.954)	0.004
Sex, male (vs. female)	0.132 (0.017–0.775	0.025
Extent of resection, lobectomy (vs. bilobectomy or pneumonectomy)	2.937 (0.308–28.510)	0.336
Pathologic TNM stage	1.153 (0.457–3.348)	0.771
Visceral pleural invasion, present (vs. absent)	0.547 (0.071–3.589)	0.529
Vascular invasion, present (vs. absent)	0.896 (0.162–5.722)	0.898
Lymphatic invasion, present (vs. absent)	0.896 (0.161–5.722)	0.898
Spread through air spaces, present (vs. absent)	6.837 (1.296–74.941)	0.021
Adjuvant therapy, yes (vs. no)	0.910 (0.111–7.808)	0.928
PB-CTCs >4 (vs. ≤4)	38.952 (5.234–396.843)	<0.001

HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis; PB, peripheral blood; CTCs, circulating tumor cells.

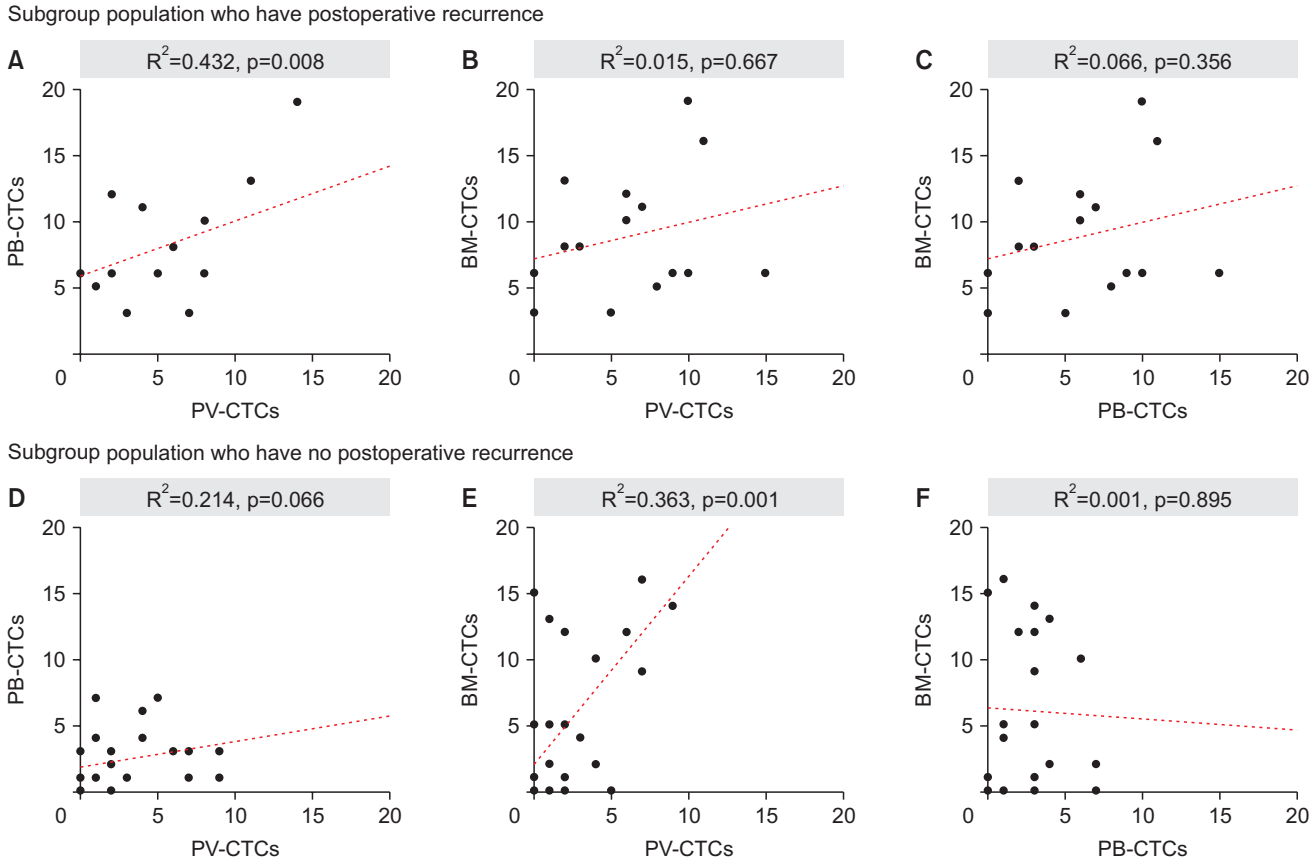

Fig. 4. Interrelationship between pulmonary vein (PV)-, peripheral blood (PB)-, and bone marrow (BM)-circulating tumor cells (CTCs) in (A–C) the subgroup of patients with postoperative recurrence, and (D–F) the subgroup of patients with no postoperative recurrence.

CD4+, and T cells [22].

Our study showed that the high PV-CTCs group was associated with pathologic nodal metastasis, but not with tumor size, location, or other pathologic factors. With a single vein draining the entire tumor basin, lung cancers are unique among solid organ tumors. Reproducible and high-yield isolation of CTCs from the PV may facilitate future studies of CTCs by helping to identify better CTC markers [12]. Reddy et al. [10] showed that the number of PV-CTCs was correlated with pathologic tumor size, but not with nodal stage or overall stage. Hashimoto et al. [9] also reported a significant increase in the CTC count in the PV responsible for drainage, especially in tumors with LI, and found that a large difference between before and after ma-

nipulation of the lung was significantly correlated with postoperative distant metastasis after curative resection in NSCLC patients.

BM has traditionally been the primary compartment in which the prognostic value of the detection of these cells has been investigated, as it is a common homing organ for tumor cells of epithelial origin [23]. A study dealing with squamous cell carcinoma of the oral cavity showed higher frequency of BM-CTCs than PB-CTCs because the half-life of CTCs in the circulation seems to be short (1–2 hours) and hence many CTCs rapidly undergo apoptosis [24,25]. According to recent observations, some BM-CTCs remain in a dormant state and may never initiate a relapse, whereas others remain in a proliferating state or escape dormancy control mechanisms to enter the cell cycle by still unknown conditions in the microenvironment or molecular alterations [26].

Compared with well-known poor prognostic factors such as high CEA, SUVmax, pathologic staging, PB-CTCs were comparable to pathologic staging, but superior to CEA or SUVmax for predicting recurrence. Many serum tumor markers have been evaluated for their clinical utility in NSCLC; however, nearly all tumor markers show a very low sensitivity. In this study, PB-CTCs showed higher sensitivity for recurrence than of CEA.

In a comparative analysis between pathologic TNM staging and CTCs, PB-CTCs and pathologic distant metastasis showed a linear correlation, meaning that patients with 8 or more PB-CTCs were more likely to have preoperative distant metastasis or postoperative recurrence. Three patients with intraoperative pleural seeding had recurrence after curative resection, in the contralateral lung, regional lymph nodes, or brain and bone. Even patients with node-negative lung cancer had recurrence when there were 8 or more PB-CTCs. When comparing patients according to whether they had 8 or more PB-CTCs or fewer than 8 PB-CTCs, there was no significant difference in recurrence patterns.

The reasons why tumors with poor prognostic factors (LI, VI, and STAS) show frequent recurrence, especially to distant sites, are unknown; however, CTCs may play a key role in this setting. In this study, patients with 4 or more PB-CTCs had more LI, VI, and STAS than those with fewer than 4 PB-CTCs. If more patients were evaluated, a correlation between these factors and the number of CTCs might be identified.

There were some limitations of this study. Among patients without CTCs from any samples, either the patients truly had zero CTCs or we may have missed alternative CTCs that might have a mesenchymal phenotype and cannot be captured using an EpCAM-based strategy, although size-based capture systems increase the detection rate of CTCs. Most currently used methods for the enrichment and identification of CTCs have deficiencies in detecting tumor cells that have lost their epithelial cell features in the course of the epithelial to mesenchymal transition. The definition of CTC positivity is unclear.

Because there were not many patients in each stage and PB-CTCs have a stronger influence on recurrence, the effects of pathologic stage were obscured by multivariate analysis and were not statistically significant. However, since the hazard ratio of pathologic stage tended to be consistent with the clinical findings, statistically significant results are expected as the number of patients increases. In terms of pleural invasion, while it is known to affect prognosis only in N0M0 tumors smaller than 3 cm [27], most of the patients in the study were in a more advanced stage, and PB-CTCs were strongly correlated with recurrence; this is thought to be why pleural invasion did not show statistical significance.

In conclusion, the presence of 4 or more PB-CTCs was an independent poor prognostic factor for RFS, and in patients with recurrence, PV-CTCs and PB-CTCs had a positive linear correlation. The evaluation of CTCs from 3 different sites might be meaningful.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Supplementary materials

Supplementary materials can be found via https://doi.org/10.5090/jcs.21.140. **Supplementary Fig 1**. (A) Scatter-

plot of peripheral blood (PB)-circulating tumor cells (CTCs) compared with pM status, (B) the receiver operating characteristic (ROC) analysis of PB-CTCs for recurrence, and (C) further details of patients with postoperative recurrence.

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