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Positive Correlation Between the Number of Circulating Tumor Cells in the Pulmonary Vein and Tumor Spread Through Air Spaces in Resected Non-small Cell Lung Cancer

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Abstract. *Background/Aim: Circulating tumor cells (CTCs) is one of the promising markers that predict dissemination and metastases. This study aimed to identify the relationship between CTCs in pulmonary vein (PuV) and spread through air space (STAS) in non-small cell lung cancers. Materials and Methods: We applied a cytology-based microfluidic platform for rare cell isolation. Twenty-four patients were enrolled. Results: The rate of CTC detection in PuV was 79.2%, and STAS was observed in 54.2% of the samples. When the definitive cut-off value was 1 CTC/1 ml, of the 14 CTC-PuV-high cases, 11 (78.6%) were STAS-positive, whereas 2 of the 10 (20.0%) CTC-PuV-low cases were STAS-positive, and the difference between the two groups was statistically significant (p=0.02). CTC-PuV-high exhibited a significantly poorer survival (p<0.01). Conclusion: The higher frequency of STAS is significantly associated with a higher number of CTCs in PuV, and the combination of STAS and CTC was significantly associated with poor prognosis.*

Non-small cell lung cancer (NSCLC) is one of the deadliest cancer types, and prognostic predictors for this type of cancer have been vigorously tested. Circulating tumor cells (CTCs) may be a useful predictor of disease prognosis and provide clinically important and comprehensive information that may accelerate research to improve survival of patients. Several clinical studies have postulated the reliability of CTCs as prognostic indicators (1-3). In addition, referring to its biological behavior, studies on the relationship between CTC detection and tumor stage demonstrated that an early-stage cancer has a smaller number of CTCs (4), thereby suggesting a possible relationship between CTCs and tumor aggressiveness.

Although many methods have been developed to isolate CTCs, there are only two basic approaches to isolate CTCs: a) methods based on detection of specific CTC surface markers and b) methods independent of specific markers, based on physical or biological properties of the CTCs. We previously reported our novel method to isolate CTCs using microfluidic device with a 3D model filter which can be used for the practical isolation of CTCs (5), without the aid of surface markers (6, 7). The microfluidic device could identify the cell configuration while recognizing epithelial lineage antigens. We consider three advantageous characteristics of this method. First, a filter made of 3D metal with 8 μm holes can capture CTCs with reduced cell damage. Therefore, morphological features of CTCs can be visualized microscopically. Second, it can be used to evaluate CTC heterogeneity, because it can detect surface marker-negative CTCs. Third, along with the determination of the CTC count, expression analyses of proteins and genes are also possible. With the availability of a fluorescent microscope and a pump, CTCs can be evaluated at a general hospital without using special equipment. We previously validated that cell size-based CTC collection from patients with different types of metastatic cancers shows enrichment of CTCs with mesenchymal and stem-like characteristics (5).

The contemporary concept of spread through air spaces (STAS) is a recent-recognized means of invasion of NSCLC.

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In 2015, the World Health Organization classification of lung cancer included the concept of STAS as a new pattern of invasion in lung adenocarcinoma (8). Since then, STAS has been the subject of extensive research regarding its value in therapeutic decision making and STAS in lung cancer has been reported to be associated with unfavorable outcomes.

Detachment of cells from the primary lesion and survival of cells for a certain period of time are indispensable for both CTCs and STAS development, and it is presumed that a common mechanism exists between CTC and STAS. However, there is an obstacle in investigating the relationship between CTC and STAS. STAS has been shown to be associated with epithelial-to-mesenchymal transition (EMT) (9, 10). Increased invasion of cancer cells stimulated by EMT could be influenced by stromal cell heterogeneity regarding overexpressed mesenchymal markers, which interfere with the search for malignant cells in the blood circulation due to the substantial change of cell surface markers.

We hypothesized that CTCs may be a predictor of STAS, and further, they may be associated with survival prognosis in combination with STAS in resected NSCLC. Our novel, surface marker-independent CTC detection method was considered to be helpful in the current analysis. We aimed to investigate the relationship between the number of CTCs in the blood obtained from the pulmonary vein (PuV) and STAS status with stage I-III NSCLC and surgical outcomes.

Materials and Methods

Patients. In this prospective cohort, we examined 24 patients (males, n=7; females, n=17) with stage I-III primary NSCLC who had previously undergone pulmonary resection at the Aichi Cancer Hospital between February 2018 and February 2019. This study was conducted in accordance with the Declaration of Helsinki. The institutional review board of Aichi Cancer Center approved this study (2018-2-32). However, only the planned patients who received thoracotomy were enrolled in this study. All patients were informed of the methods and provided consent to the study protocol prior to pulmonary resection.

All patients with NSCLC underwent lobectomy and lymph node dissection *via* thoracotomy, specifically a vertical muscle-sparing thoracotomy (11). During lobectomy, after ligation of the central side of the PuV at the proximal side of the heart, we immediately punctured the PuV using an 18-gauge needle and collected an average of 6.0±2.9 ml blood (range=1-10 ml). Also, an average of 7.5±4.8 ml blood from PeA (range=2.5-26 ml) was collected during the operation. Data postoperatively collected from patient records included age, sex, routine perioperative laboratory data [carcinoembryonic antigen or, D-dimer; and prognostic nutrition index (calculated using the following formula=serum albumin levels (g/dl) × 10 + total lymphocyte count (per mm^3) ×0.005)], which is associated with prognosis of surgically resected early NSCLC (12), smoking status (pack-years), and maximum standard uptake value on a positron emission tomography scan.

The histology of NSCLC was estimated based on the eighth Union for International Cancer Control criteria and STAS was diagnosed according to the 2015 World Health Organization classification of lung tumors by at least two pathologists (8, 13). Pathological staging was determined according to the eighth edition of the Tumor-Node-Metastasis classification (14). Disease-free survival was defined as the period between the date of pulmonary resection and the date of recurrence, and in the absence of cancer related death.

The methods for the analysis of each mutation [epidermal growth factor receptor (*EGFR*), Ki-ras2 Kirsten rat sarcoma (*KRAS*), and other mutations in genes including *ALK, HER2, BRAF, ROS1*, and *MET* have been previously described (15). *EGFR* (exons 18-21) mutations were identified using the cycleave polymerase chain reaction method. *KRAS* (exons 2-3), *BRAF* (exons 11-15), *HER2* (exon 20), and *MET* (exon 14) mutations were assessed using fragment analysis, and the results were validated by direct sequencing. *ALK* and *ROS1* mutations were first screened using immunohistochemistry, and the final confirmation was performed using fluorescence *in situ* hybridization.

Analyses of CTCs. The CTC detection system was introduced in a previous study (5, 16, 17). A microfluidic device with a 3D metal filter for the enrichment of rare cells was developed using an injection molding technology. Briefly, an 8 μm pore in the lower layer and a 30 μm-sized cell capture hole in the upper layer produced using the micro-fabrication technology in combination with electroforming processes could be realized to capture CTCs with less cell damage (Optnics Precision Co. Ltd. Tochigi, Japan), as described previously (5). Rare cells with a diameter of more than 10 μm trapped in the filter were then quickly transferred to a glass slide and immediately fixed in 95% ethanol (for more than 1 h to one week) for Papanicolaou staining (Sakura Fintec, Tokyo, Japan) (Figure 1A). Another aliquot was fixed in 95% ethanol, followed by 10% buffered formalin for 20 min for cytokeratin immunocytochemistry (Figure 1B and C). Representative megakaryocytes from PuV were stained by Papanicolaou as previously published (Figure 1D) (5).

Statistical analyses. Data were analyzed using the Statistical Package for the Social Sciences software (version 25.0; SPSS Inc., Chicago, IL, USA). We investigated the cut-off value of CTC number to identify the presence of STAS using the receiver operating characteristic (ROC) curve and measured the area under the receiver operating characteristics. The statistical significance of the numerical differences between groups was determined using the Mann–Whitney *U*-test. The Kaplan–Meier method was used to analyze survival rates in patient subsets; differences in survival between the groups were assessed using the log-rank test. A p-value <0.05 was considered significant.

Results

Twenty-four patients were enrolled in the study. Patient baseline characteristics are summarized in Table I. Seventeen patients were female (70.8%) and seven were male (29.2%). The mean age was 70 years, ranging from 54 to 79 years. The most frequent histological tumor type diagnosed was adenocarcinoma (n=15, 62.5%), followed by squamous (n=5, 20.9%), adenosquamous (n=2, 8.3%), and large cell carcinoma (n=2, 8.3%). The number of patients diagnosed with the pathological stages IIIA, IIB, IA, IB, and IIA was nine (37.5%), six (25.0%), five (20.8%), three (12.5%), and one (4.2%), respectively.

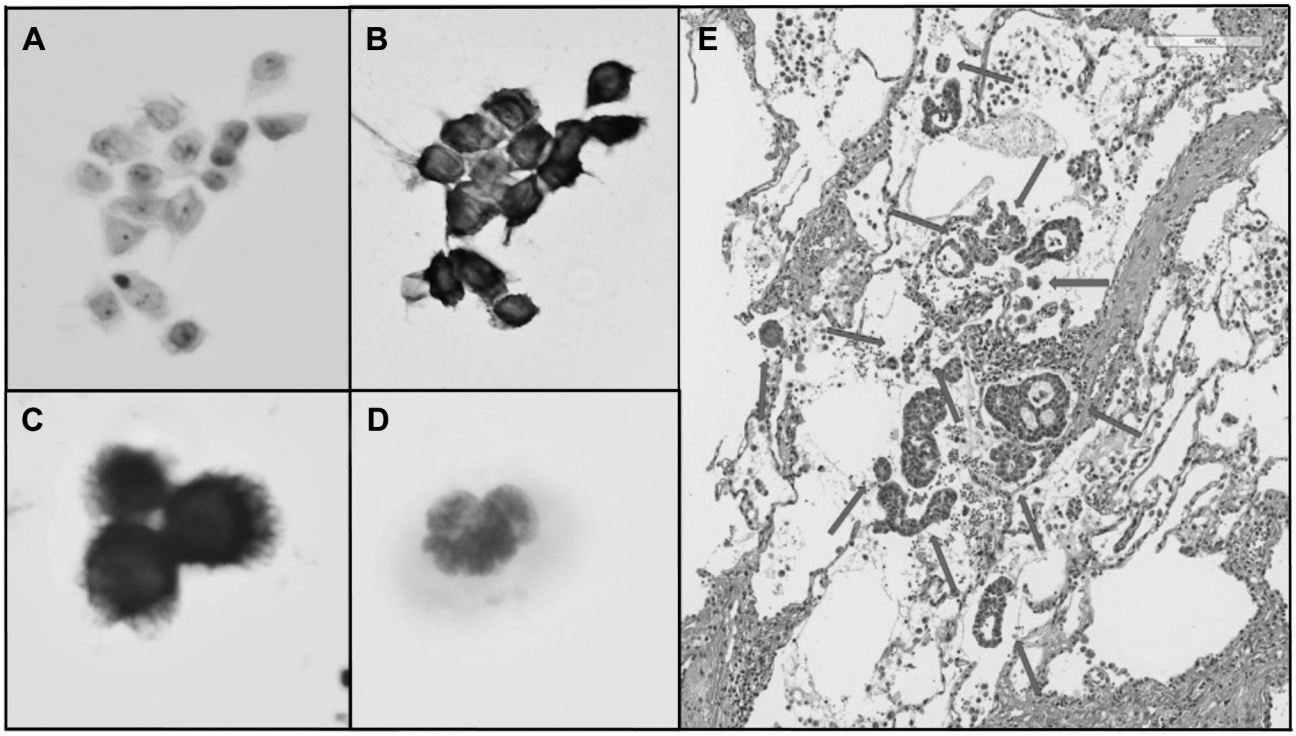

Figure 1. *Representative images of cytological detection of circulating tumor cells in the pulmonary vein. (A) Large cluster stained using Papanicolaou, (B) large cluster stained using cytokeratin, (C) small cluster, (D) megakaryocyte stained using Papanicolaou, and (E) numerous circulating tumor cell spread through air spaces (red arrows) in surgically resected specimens.*

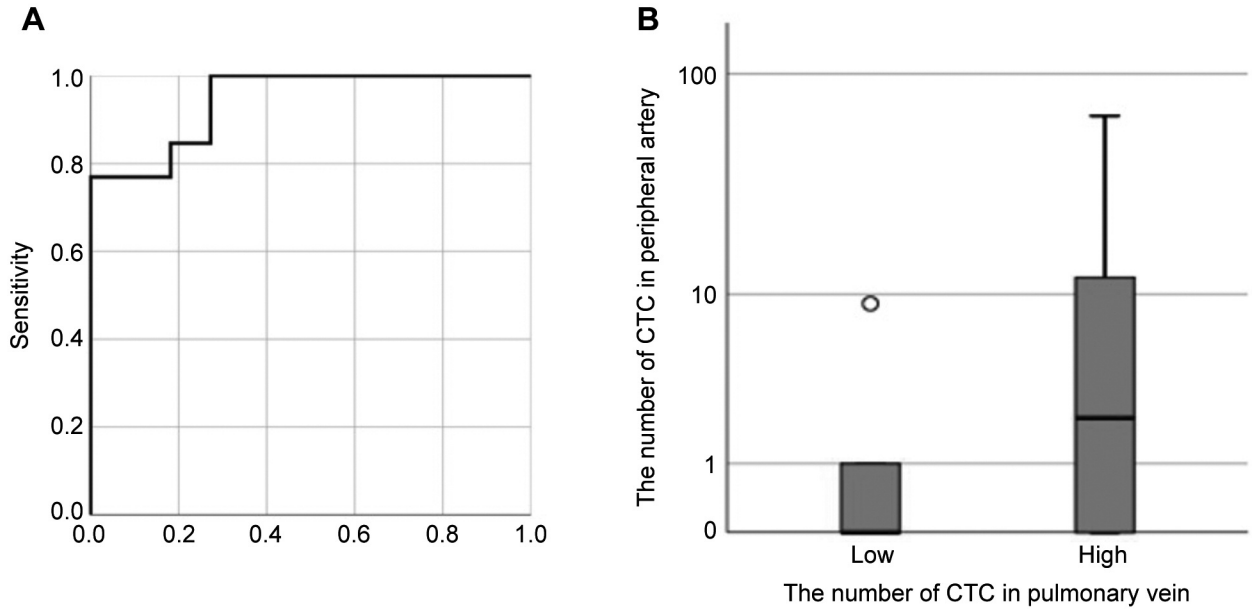

Figure 2. *The correlation between CTCs and spread through air spaces. (A) ROC curve analyses for the emergence of spread through air according to the number of CTCs. (B) Quantitative analysis of the number of CTCs in the peripheral artery according to the ROC curves. ROC: Receiver operating characteristic; CTC: circulating tumor cell.*

Table I. *Clinicopathological characteristics*.

Characteristics	PuV-CTC-HG n=14	PuV-CTC-LG n=10	*p*-Value
Age (median)	70	71	0.66
Gender, male	12 (85.7%)	5 (50.0%)	0.06
Smoking, pack-year, (median, IQR)	31.5 (2.7-45.3)	37.5 (0-73.9)	0.84
Carcinoembryonic antigen (ng/ml, median, IQR)	4.1 (2.4-7.8)	4.3 (2.1-9.6)	0.06
Prognostic nutrition index (≥50)	7 (50.0%)	4 (40%)	0.64
D-dimer (median, IQR)	0.5 (0.5-1.5)	0.5 (0.5-0.9)	0.62
Maximum standard uptake on PET (median, IQR)	15.0 (9.2-24.2)	17.3 (4.3-19.7)	0.93
Histology, adenocarcinoma	11 (78.6%)	4 (40.0%)	0.06
Pathological invasive size (mm)	36.0 (22.0-47.3)	29.0 (21.5-42.0)	0.56
c-Stage			0.01
IA	2 (14.3%)	5 (50.0%)	
IB	0	1 (10.0%)	
IIA	2 (14.3%)	2 (20.0%)	
IIB	7 (50.0%)	2 (20.0%)	
IIIA	3 (21.4%)	0	
p-Stage			<0.01
IA	0	5 (50.0%)	
IB	2 (14.3%)	1 (10.0%)	
IIA	0	1 (10.0%)	
IIB	4 (28.6%)	2 (20.0%)	
IIIA	8 (57.1%)	1 (10.0%)	
Genomic mutations			0.90
EGFR	5 (35.7%)	4 (40.0%)	
KRAS	2 (14.3%)	1 (10.0%)	
MET	1 (7.1%)	1 (10.0%)	
No mutations	6 (42.9%)	4 (40.0%)	
Adjuvant chemotherapy	3 (21.4%)	1 (10.0%)	0.42
Megakaryocytes	24.5 (17.5-46.0)	19.0 (7.8-65.0)	0.38

PuV-CTC-HG/LG: Pulmonary vein-circulating tumor cells-high/low group; PET: positron emission tomography; EGFR: epidermal growth factor receptor; KRAS: Ki-ras2 Kirsten rat sarcoma; IQR: interquartile range.

Detection of CTCs in the PuV and peripheral artery (PeA). The detection rate of CTCs was higher in the PuV (n= 9, 79.2%) than in the PeA (n=13, 54.2%) but the difference was not statistically significant (p=0.07). The mean CTC number was higher in the PuV (34.5; interquartile range=21.5-88.3) than in the PeA (1.0; interquartile range=0-3.8) (p<0.01).

Pathological significance of the PuV-CTC number. Tumor STAS was pathologically proven in 13 (54.2%) resected NSCLCs as shown in Figure 1E. When we assessed the definitive performance of PuV-CTC number using a ROC curve; the area under the receiver operating characteristics for the diagnosis of STAS was 0.94 (Figure 2A). Based on the number of detected CTCs, our cases were categorized into two subgroups, CTC-PuV-high (≥1/1 ml) and CTC-PuV-low (<1/1 ml) according to the calculated cut-off value using the ROC curves (Figure 2B). Of the 14 CTC-PuV-high cases, 11 were STAS-positive (11/14, 78.6%), whereas 2 of the 10 CTC-PuV-low cases were STAS-positive (2/10, 20.0%), and the difference between the two groups was significant (p=0.02). The rate of pathological stage migration tended to be higher in the PuV-CTC-high group (6/14, 42.9%) than in the PuV-CTC-low group (1/10, 10.0%), however, no statistically significant difference was observed (p=0.09).

Clinical outcomes. Postoperative median follow-up duration was 21.3 months (range=17.3-22.8 months). Only three patients (12.0%) received adjuvant chemotherapy. One patient (4.2%) died of cancer, one patient (4.2%) died from a cause other than cancer, and 11 patients (45.8%) experienced recurrence during this period. The 2-year disease-free survival was shorter in the PuV-CTC-high group (24.1%) than in the PuV-CTC-low group (75.0%), however, the difference was not significant (p<0.01) (Figure 3).

Discussion

In this study, we prospectively investigated whether the CTC number obtained from the PuV and PeA was clinically associated with clinicopathological characteristics and

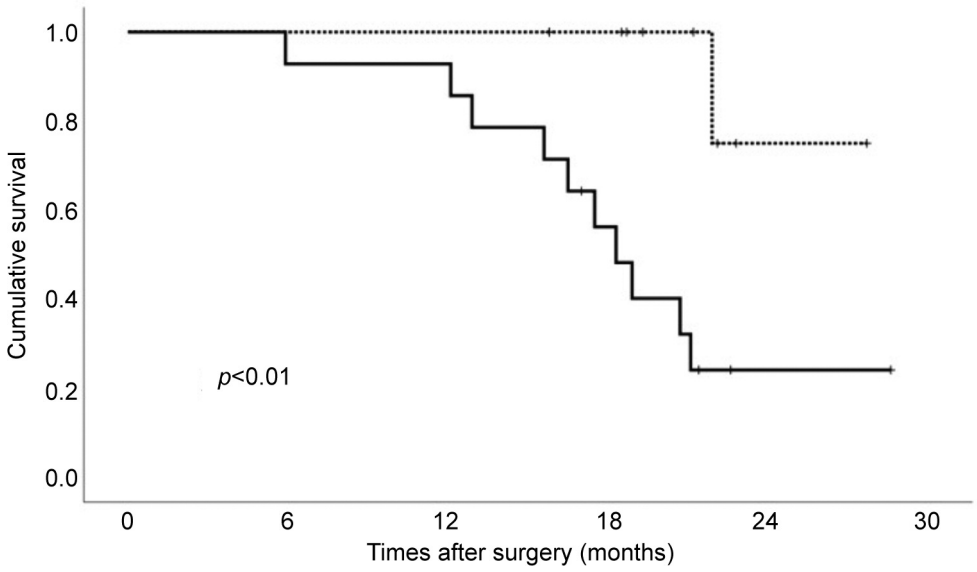

Figure 3. *One-year overall survival after pulmonary resection of patients. Dotted line) low-expression of circulating tumor cells; and Black line) high-expression of circulating tumor cells.*

surgical outcomes. Our key findings can be summarized as follows: 1) the detection sensitivity and mean number of CTCs in the PuV were higher than those in the PeA; 2) STAS was more frequently proven on histopathologic diagnosis in patients with CTC number ≥1/1 ml, (78.6%); 3) pathologic stage migration was relatively higher in the PuV-CTC-high group than in the PuV-CTC-low group; 4) the higher number of CTCs was associated with poor prognosis. These findings imply that the detection of CTCs in the PuV provides advantages since liquid biopsy efficiently predicts postoperative cancer recurrence in patients undergoing surgical resection with curative intent. In addition, the CTC number in both PeA (≥1/10 ml) and PuV (≥1/1 ml) showed a robust relationship with STAS (1/1, 100%).

With regard to the detected cell number, CTC levels obtained from PuV were higher than those from PeA. Since CTC collection from the PuV is highly invasive, however we were able to find a sufficient number of CTCs in the PuV compared to the peripheral blood vessels. The main reason why blood collection from the PuV is suitable for CTC collection is that the characteristics of the PuV blood represent the direct flow from cancer. Tumor cell size, including that of CTCs, ranges from 9 to 30 mm (18). Blood capillaries have a diameter ranging 3-8 mm and thus quite a large number of CTCs is presumed to be caught in their lumen (19, 20). Therefore, not only the number of CTCs detached from tumors, but also the interaction between local blood vessels and CTCs and other factors (*e.g.*, the rate of blood shunt flow) determine the number of CTCs obtained from peripheral blood samples. In peripheral blood vessels, CTCs and endothelial cells are known to be bound *via* adhesion factors, and that lymphocytes and platelets promote the formation of traps in peripheral tissues (21).

Another reason why the number of CTCs detected from PuV is higher than that of PeA is that the cell size changes according to the blood flow. CTCs released into the blood undergo physical changes such as shrinking in size during the blood flow. Okano *et al*. reported that the size of circulating mice tumor cells became small as the liquid flow increases in experimental models (22). Blood flow was faster in PeA than in PuV, and it is possible that pore passage occurred due to the reduction in the size of CTCs. In order to overcome these obstacles, separation methods based on characteristics other than cell size should be applied in combination, for example, using density-based separation and/or specific electrical properties of the CTCs.

With the surface markers-independent CTC detection method, we demonstrated that the proportion of STAS in NSCLCs with ≥1 CTCs/1 ml was significantly higher than that with <1 CTCs/1 ml. The patients with CTC numbers ≥1/1 ml in the PuV had a tendency of poorer DFS during this short-term follow-up period. Importantly, although a significant difference was not obtained, pathological stage migration was identified in 42.9% of NSCLCs with ≥1 CTCs/1 ml, whereas it was 10.0% in those with <1 CTCs/1 ml (p=0.09). After the announcement of the concept of STAS according to the WHO classification of lung tumors in 2015, several authors have reported the association of STAS with malignant invasiveness,

occult lymph node metastases, and poor prognosis in adenocarcinoma, even in stage I (23, 24). Jia *et al*. reported that STAS-positive cases were more likely to show low E-cadherin expression and high vimentin expression based on the analysis of 303 lung adenocarcinomas and 121 squamous cell carcinomas (25). The mechanism of EMT, by which adherent epithelial cells are thought to acquire migratory capabilities, includes the combined activation of proteases, which compromise the integrity of the basement membrane and the extracellular matrix, leading to the intravasation of tumor cells into the blood steam (26). Regarding cancer metastasis and EMT, Rhim *et al*. reported that EMT and malignant cell dissemination might occur early during the development of cancer (27). The epithelial and mesenchymal heterogeneity in CTCs provides evidence that EMT may not only permit escape from the primary tumor and invasion, but may also increase tumor-initiating properties. It is uncertain whether EMT can explain all the relationships between STAS and CTCs, but it is impossible to accurately capture the CTCs in this study. Therefore, further accumulation of data about CTCs that have undergone EMT changes will be needed.

There were several limitations to this study. First, although this study was prospective in nature, the sample size was small, and included a selection bias to a small extent. If there is a crucial difference in bias, the results of this study might not be valid. Second, our institutional review board permitted CTC collection under only thoracotomy in this study. We could obtain CTCs from a limited number of patients. Our institutional review board recognized that the 18-gauge needle aspiration of the blood from the PV after ligation of the central side was considered a smooth transition under thoracotomy compared to thoracoscopic surgery, and permitted this protocol only in the patients who underwent thoracotomy with a necessity for reducing intraoperative bleeding risk owing to the availability of a short time. Third, tumors larger than 30 mm, clinical lymph node positivity (cN1-2), neoadjuvant chemotherapy, and patient motivation were indications for thoracotomy in our institution. This study included seven patients at the clinical stages IA (29.2%, 7/24). Fourth, this study was conducted in a single institution. Thus, further large-scale multi-center prospective studies are desirable.

Conclusion

In summary, we found that pulmonary venous blood is an appropriate sample for analyzing CTC in NSCLC. STAS was pathologically proven in 54.2% of patients. The higher the proportion of STAS in primary tumors, the more abundant the CTC number. A high CTC number (≥1/1 ml) in the pulmonary vein was significantly associated with a poor prognosis.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

Conceptualization, H.K. and K.M.; methodology, H.K. and H.N.; software, K.M; validation, YT., T.N., and E.S.; formal analysis, H.N. and N.S.; investigation, H.K. and K.M.; resources, N.S. and T.N.; data curation, Y.T.; writing – original draft preparation, H.K.; writing – review and editing, K.M.; visualization, S.F.; supervision, Y.Y.; project administration, H.N.

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