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Significant increase in circulating tumour cells in pulmonary venous blood during surgical manipulation in patients with primary lung cancer[†]

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Abstract

OBJECTIVES: Circulating tumour cells (CTCs) are tumour cells shed from a primary tumour and circulate in the peripheral blood after passing through the drainage vein. In previous studies, we showed that high numbers of CTCs were detected in the drainage pulmonary venous blood of most patients with resectable primary lung cancer, whereas only low numbers of CTCs were detected in the peripheral blood of some patients. Accordingly, this prospective study was conducted to assess changes in CTCs in the drainage pulmonary vein (PV) during lung cancer surgery.

METHODS: A total of 30 consecutive peripheral-type primary lung cancer patients who underwent lobectomy (or right upper and middle bilobectomy) through open thoracotomy were included. For each patient, 2.5 ml of blood was sampled from the lobar PV of the primary tumour site before and after surgical manipulation for lobectomy. The CTCs were evaluated quantitatively with the CellSearch® system.

RESULTS: Before surgical manipulation, CTCs were detected in PV blood in the majority of patients (22 of 30, 73.3%), although CTCs were detected in peripheral blood in only two patients (6.7%). The median number of CTCs in the PV (pvCTC-count) before surgical manipulation was 4.0 cells/2.5 ml, and there was no significant correlation between pvPV-count and any clinicopathological characteristic, including tumour size, progression and histological type. After surgical manipulation, at the time of completion of the lobectomy, the pvCTC-count significantly increased (median, 60.0 cells/2.5 ml; $P = 0.001$). The increase in pvCTC-count was significantly associated with microscopic lymphatic tumour invasion (ly); pvCTC-count significantly increased in ly-positive patients (pvCTC-count before and after surgical manipulation, 4.0 and 90.5 cells/2.5 ml, respectively; $P = 0.006$), but not in ly-negative patients (3.5 and 7.0 cells/2.5 ml, respectively; $P = 0.153$). The increase in pvCTC-count was not significantly associated with any other clinicopathological factor or with any surgical procedure, including the sequence of vessel interruption.

CONCLUSIONS: We documented a significant increase in CTC count in drainage PV blood after surgical manipulation, especially in tumours with lymphatic invasion. We are awaiting survival data at 5 year follow-up examination, which may provide clinical significance of the pvCTC-count.

Keywords: Lung cancer • Circulating tumour cell • Pulmonary vein • Surgical manipulation

INTRODUCTION

Distant metastases are detected clinically in ~40% of patients with primary lung cancer [1], and distant metastasis may frequently develop during treatment or follow-up after completion of treatment even in patients without distant metastasis at the time of diagnosis. Thus, in most primary lung cancer patients, tumour cells originating from the primary tumour may circulate in peripheral blood with or without clinically detectable distant metastasis, and detection of circulating tumour cells (CTCs) may be important in the diagnosis and therapy of primary lung cancer [2]. However, in spite of many efforts to develop a sensitive detection system for rare CTCs contaminating the peripheral blood, the clinical

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significance of CTCs has not been established, mainly due to lack of accuracy and reproducibility in detection of CTCs [3, 4].

Recently, the CellSearch® system (Veridex LLC, Raritan, NJ, USA) has been developed, in which CTCs are immunomagnetically captured with an antibody against epithelial cell adhesion molecule (EpCAM) [3, 4]. Based on accumulating data supporting the accuracy and precision for evaluating CTCs [5–8], the CTC-test using the CellSearch system has been approved in the USA by the Food and Drug Administration (FDA) for monitoring of blood from metastatic breast, colon and prostate cancer patients.

In primary lung cancer patients, we conducted a series of prospective studies to assess the clinical significance of CTCs evaluated with the CellSearch system [9–11]. First, we demonstrated that CTCs were detected in peripheral blood in 30.6% of primary lung cancer patients and that the presence of CTCs in peripheral blood was significantly associated with the presence of clinically detectable distant metastasis [9]. Second, we demonstrated that the CTC-test provided significant prognostic value in small-cell lung cancer patients [10]. In parallel with prospective studies on CTCs in peripheral blood (periCTCs) of primary lung cancer patients, we initiated a prospective study on CTCs in pulmonary venous blood (pvCTCs), because tumour cells may separate from the primary tumour and circulate after passing through the drainage pulmonary vein (PV). In a preliminary pvCTCs study of 30 consecutive patients who underwent surgery for primary lung cancer [11], we demonstrated that CTCs were detected in most (29 of 30, 96.7%) patients, and that the mean and median numbers of CTCs in PV blood (pvCTC-count) were 1195 and 81 (per 7.5 ml of PV blood), respectively. These results suggest that a large number of tumour cells originating from the primary tumour may pass through the drainage PV and may develop into distant metastases, and this process can be accelerated by surgical manipulation. Thus, we conducted a prospective study to compare pvCTCs before and after surgical manipulation.

MATERIALS AND METHODS

Patients

Patients who were admitted for lobectomy or bilobectomy (right upper and middle lobectomy, only) through open thoracotomy for peripheral-type primary lung cancer at the Department of Thoracic Surgery, Hyogo College of Medicine (HCM) Hospital, were eligible. From August 2009 to October 2010, a total of 30 patients provided written informed consent and were enrolled in the study (Table 1). Complete clinical data, including history, physical examination and radiographic studies, were collected. For the evaluation of tumour progression, whole-body computed tomography, brain magnetic resonance imaging and positron emission tomography scanning were routinely performed. The clinical (c-) stage and pathological (p-) stage were determined according to

Table 1: Patient characteristics

Characteristic		No. of patients	Percentage
Sex	Male/female	18/12	(60.0%/40.0%)
Age	Mean, median, range (years)	68.0, 67.0, 41–80	
Histology	Adenocarcinoma	22	(73.3%)
	Squamous cell carcinoma	6	(20.0%)
	Others	2	(6.7%)
Site of primary tumour	Right upper lobe	16	(53.3%)
	Right middle lobe	2	(6.7%)
	Right lower lobe	5	(16.7%)
	Left upper lobe	6	(20.0%)
	Left lower lobe	1	(3.3%)
Mode of operation	Lobectomy	28	(93.3%)
	Bilobectomy (RUML)	2	(6.7%)
Sequence of vessel ligation	PA → PV	21	(70.0%)
	PV → PA	9	(30.0%)
Pathological stage	I	17	(56.7%)
	II	8	(26.7%)
	III	3	(10.0%)
	IV	2	(6.7%)
Pathological T factor (pT)	1	6	(20.0%
	2	18	(60.0%)
	3–4	6	(20.0%)
Pathological N factor (pN)	0	22	(73.3%)
	1	5	(16.7%)
	2	3	(10.0%)
Diameter of primary tumour	Mean, median, range	3.0, 3.3, 1.5–6.0	
Pleural invasion of primary tumour	Negative	13	(43.3%)
	Positive	17	(56.7%)
Lymphatic invasion of primary tumour	Positive	10	(33.3%)
	Negative	20	(66.7%)
Vessel invasion of primary tumour	Negative	8	(26.7%)
	Positive	22	(73.3%)
Total		30	(100%)

PA: pulmonary artery; PV: pulmonary vein; RUML: right upper and middle lobes.

the current tumour–node–metastasis (TNM) classification as revised in 2009 [12]. Pleural invasion (pl), lymphatic invasion (ly) and vessel invasion (v) of primary tumour in the resected lung were evaluated under light microscopy. The study was approved by the Institutional Review Board of HCM.

Most patients (27 of 30, 90.0%) had c-stage I ($n = 22$) or c-stage II disease ($n = 5$), and all patients achieved microscopic complete resection by lobectomy or bilobectomy with nodal dissection through open thoracotomy. After pathological examination of resected specimens, three patients changed from c-stage I–II to p-stage III–VI (one from c-stage I to p-stage IIIA owing to T3N1 disease, one from c-stage I to p-stage IV owing to intrapulmonary metastases, and one from c-stage II to p-stage IIIA owing to mediastinal nodal metastases), and one patient changed from c-stage III to p-stage II due to a lack of pathological mediastinal nodal metastases, whereas preoperative computed tomography showed enlarged mediastial nodes with abnormal fludeoxyglucose uptake. Finally, 17 patients (56.7%) and eight patients (26.7%) had p-stage I and II disease, respectively; only five patients (16.7%) had p-stage III–IV disease.

Surgical procedure and blood sampling

After induction of general anaesthesia, a polyethylene catheter was inserted into the radial artery for monitoring blood pressure during surgery, and 7.5 ml of peripheral blood was drawn from the catheter immediately before thoracotomy and was collected in a CellSaver tube (Veridex LLC). After thoracotomy, the drainage lobar PV, i.e. the pulmonary vein of the same lobe as the primary tumour, was first exposed and punctured with a 23 gauge needle, and 2.5 ml of blood was drawn from the PV prior to subsequent surgical manipulation for lobectomy. In principle, the lobar pulmonary arteries (PAs) were interrupted first (PA first) for lobectomy, followed by interruption of the PV, and finally the bronchus was dissected and closed. However, the order of vessel interruption (PA first or vice versa, i.e. PV first) was ultimately chosen by the performing surgeons, and PA first was performed in 21 (70.0%) of the 30 patients. There was no significant difference in the sequence of vessel interruption according to the site of the primary tumour; PA first was performed in 10 (62.5%) of 16 right upper lung tumours, in two (100%) of two right middle lobe tumours, in three (75.0%) of four right lower lobe tumours, in five (71.4%) of seven left upper lobe tumours and in one (100%) of one left lower lobe tumour, respectively. Immediately after completion of lobectomy, 2.5 ml of blood was drawn from the resected drainage PV. For each blood sample taken from the PV, 5.0 ml of the dilution buffer was added, and a total of 7.5 ml diluted sample was collected in a CellSaver tube. All blood samples were stored at room temperature, and were provided for preparation of CTCs within 72 h after sampling.

Evaluation of circulating tumour cells

Circulating tumour cells were captured and evaluated quantitatively using a semi-automated system, the CellSearch system, following the manufacturer's protocol as described previously [9–11]. In brief, CTCs were immunomagnetically captured using ferroparticles coupled to a monoclonal antibody against the epithelial cell adhesion molecule (EpCAM), and the CTC-enriched samples were then stained with 4′,6-diamidino-2-phenylindole (DAPI) and an anti-cytokeratin antibody conjugated with phycoerythrin. Contaminated white blood cells were excluded by negative selection for CD45. Stained cells were then analysed under a fluorescence microscope using the CellTracks Analyzer II (Veridex LLC). The criteria for each cell to be defined as a CTC are as follows: round to oval morphology; a visible DAPI-positive nucleus; positive cytokeratin staining in the cytoplasm; and negative staining for CD45. All evaluations were performed without knowledge of the clinical characteristics of the patients. The number of CTCs per 7.5 ml of peripheral blood was represented as the periCTC-count; the number of CTCs per 2.5 ml of PV blood was represented as the pvCTC-count.

Statistics

The sample size ($n = 30$) was not predetermined based on statistical estimation, but was the number of total cases enrolled in the study period. Counts were compared by the χ^2 test. Continuous data were compared using Student's unpaired *t*-test for comparison between two groups or analysis of variance (ANOVA) for comparison among three or more groups, if the distribution of samples was normal; if the sample distribution was asymmetrical, continuous data were compared using non-parametric tests (Mann–Whitney *U*-test for comparison between two groups and Kruskal–Wallis test for comparison among three or more groups). A non-parametric test (Wilcoxon's signed rank test) was used to compare the difference between pvCTC-counts before and after manipulation of each patient. Differences were considered significant when the *P*-value was <0.05. All statistical manipulations were performed using the IBM SPSS version 20 (IBM Japan, Tokyo, Japan).

RESULTS

Circulating tumour cells in peripheral blood and pulmonary venous blood before surgical manipulation

Before surgical manipulation, CTCs were detected in PV blood in the majority of patients (22 of 30, 73.3%), and the median pvCTC-count was 4.0 cells/2.5 ml (range, 0–1122 cells/2.5 ml). There was no significant correlation between the pre-pvCTC-count and any clinicopathological characteristic, including tumour size, progression and histological type (Table 2).

No CTCs were detected in peripheral blood in most patients (28 of 30, 93.3%). Only one CTC was detected in 7.5 ml of peripheral blood in two patients (6.7%), one with p-stage IIA (T2aN1M0) squamous cell carcinoma and the other with p-stage IIIA (T3N1M0) adenocarcinoma; the pvCTC-counts for these patients were 3 and 12 cells/2.5 ml, respectively.

Circulating tumour cells in pulmonary venous blood after surgical manipulation

After surgical manipulation, the pvCTC-count was significantly increased (median, 60.0 cells/2.5 ml; range, 0–1855 cells/2.5 ml; $P = 0.001$), and the median increase in the pvCTC-count was 45.5 cells/2.5 ml (Fig. 1). In four patients, the pvCTC-count decreased after surgical manipulation. All four patients had adenocarcinoma

Table 2: Patient characteristics and pvCTC-count

		pvCTC-count (median, per 2.5 ml of blood)					
		Before		After		Change	
Sex							
Male	$n = 18$	4.8	$P = 0.602$	64.0	$P = 0.850$	+45.5	$P = 0.465$
Female	$n = 12$	4.0		40.5		+36.0	
Age							
Lower (<67 years)	$n = 14$	3.8	$P = 0.580$	76.0	$P = 0.208$	+58.5	$P = 0.277$
Higher (≥67 years)	$n = 16$	6.0		39.0		+29.0	
Histology							
Adenocarcinoma	$n = 22$	5.7	$P = 0.118$	39.0	$P = 0.304$	+29.0	$P = 0.162$
Non-adenocarcinoma	$n = 8$	3.0		64.5		+54.5	
Side							
Right	$n = 23$	4.3	$P = 0.441$	35.0	$P = 0.266$	+24.0	$P = 0.107$
Left	$n = 7$	3.0		70.0		+61.0	
Lobe							
Upper	$n = 22$	3.6	$P = 0.426$	60.0	$P = 0.790$	+45.5	$P = 0.991$
Middle	$n = 2$	22.0		278.0		+256.0	
Lower	$n = 6$	6.0		43.5		+31.0	
Sequence of vessel ligation							
PA → PV	$n = 21$	3.9	$P = 0.400$	70.0	$P = 0.230$	+56.0	$P = 0.193$
PV → PA	$n = 9$	5.0		38.0		+33.0	
Duration of operation							
<123 min	$n = 15$	5.0	$P = 0.390$	61.0	$P = 0.813$	+46.0	$P = 0.870$
≥123 min	$n = 15$	3.0		59.0		+45.0	
Blood loss during operation							
<50 ml	$n = 14$	4.4	$P = 0.759$	58.0	$P = 0.608$	+48.5	$P = 0.473$
≥50 ml	$n = 16$	3.8		64.0		+26.0	
Pathological tumour factor (pT)							
1	$n = 6$	4.5	$P = 0.820$	12.5	$P = 0.321$	+4.0	$P = 0.143$
2–4	$n = 24$	4.9		65.0		+51.0	
Primary tumour diameter							
<30 mm	$n = 13$	4.3	$P = 0.967$	23.0	$P = 0.385$	+14.0	$P = 0.198$
≥30 mm	$n = 17$	4.0		69.0		+52.7	
Primary tumour pleural invasion (pl)							
Negative (pl0)	$n = 13$	3.8	$P = 0.592$	55.0	$P = 0.432$	+41.0	$P = 0.621$
Positive (pl1–3)	$n = 17$	5.5		80.0		+51.0	
Primary tumour lymphatic invasion (ly)							
Negative (ly0)	$n = 10$	3.5	$P = 0.650$	7.0	$P = 0.028$	+2.5	$P = 0.043$
Positive (ly1–3)	$n = 20$	4.0		90.5		+53.5	
Primary tumour vessel invasion (v)							
Negative (v0)	$n = 8$	4.7	$P = 0.629$	58.0	$P = 0.700$	+48.0	$P = 0.629$
Positive (v1–3)	$n = 22$	3.8		64.0		+26.5	
Pathological nodal factor (pN)							
0	$n = 22$	4.0	$P = 0.629$	66.5	$P = 0.801$	+45.5	$P = 0.945$
1–2	$n = 8$	8.0		39.5		+29.0	
Total	$n = 30$	4.0		60.0		+45.5	

PA: pulmonary artery; PV: pulmonary vein; pvCTC-count: median number of circulating tumour cells in the pulmonary vein.

originating in the right lung (upper lobe in two patients, middle lobe in one patient and lower lobe in one patient), but no other specific characteristics across these four patients, such as lymphatic invasion, were documented.

Association between the increase in pvCTC-count and each clinico-pathological parameter was analyzed (Table 2 and Figs 2–5). The increase in the pvCTC-count was significantly associated with microscopic lymphatic invasion ($P = 0.043$; Table 2); the pvCTC-count was significantly increased in tumours with positive lymphatic invasion (pvCTC-count before and after surgical manipulation, 4.0 and 90.5 cells/2.5 ml, respectively; $P = 0.006$), but not in tumours without lymphatic invasion (3.5 and 7.0 cells/2.5 ml, respectively; $P = 0.153$; Fig. 4). The increase in pvCTC-count was not significantly associated with any other clinicopathological factor, including histology, primary tumour site and nodal status.

After surgical manipulation, the pvCTC-count increased significantly regardless of the duration of the operation (Table 2). Moreover, the increase in pvCTC-count was not significantly associated with blood loss during surgery, the mode of operation (lobectomy or bilobectomy) or the sequence of vessel ligation (PA-first or PV-first; Fig. 6).

DISCUSSION

In the present study, we evaluated quantitatively CTCs sampled before surgical manipulation of lung resection in both peripheral blood and drainage PV blood using the CellSearch system in patients with resectable primary lung cancer, and showed that a higher number of CTCs (median, 4.0 cells/2.5 ml of PV blood;

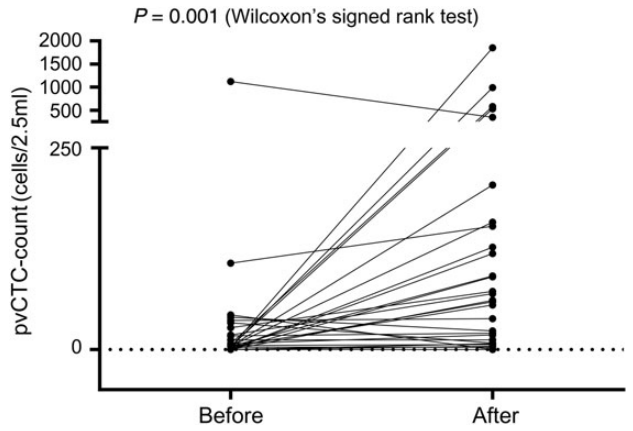

Figure 1: Comparison of the number of circulating tumour cells in 2.5 ml of drainage pulmonary venous blood (pvCTC-count) before and after surgical manipulation for all patients.

range, 0–1122 cells/2.5 ml) were detected in PV blood of most patients (77.3%), whereas only one CTC per 7.5 ml) was detected in peripheral blood of 6.7% of patients. Sienel and coworkers also evaluated CTCs in PV blood before surgical manipulation for lung resection using another system, in which mononuclear cells, including CTCs, were isolated with density gradient centrifugation and CTCs were detected by immunohistochemical staining using an antibody against cytokeratins (CKs) 8, 18 and 1, and CTCs were detected in 18% (11 of 62) of patients with resectable non-small-cell lung cancer (NSCLC) [13]. The incidence of the presence of CTCs in PV blood seemed to be lower than that documented in our present study (73.3%), which may suggest superiority of the CellSearch system used in our study. Sienel and coworkers showed that the presence of CTCs in PV blood was significantly associated with a poor prognosis [13], but it remains unknown whether these tumour cells may develop into distant metastases and lead to death from cancer. Accordingly, the biological and clinical significance of the presence of tumour cells in PV blood remains to be

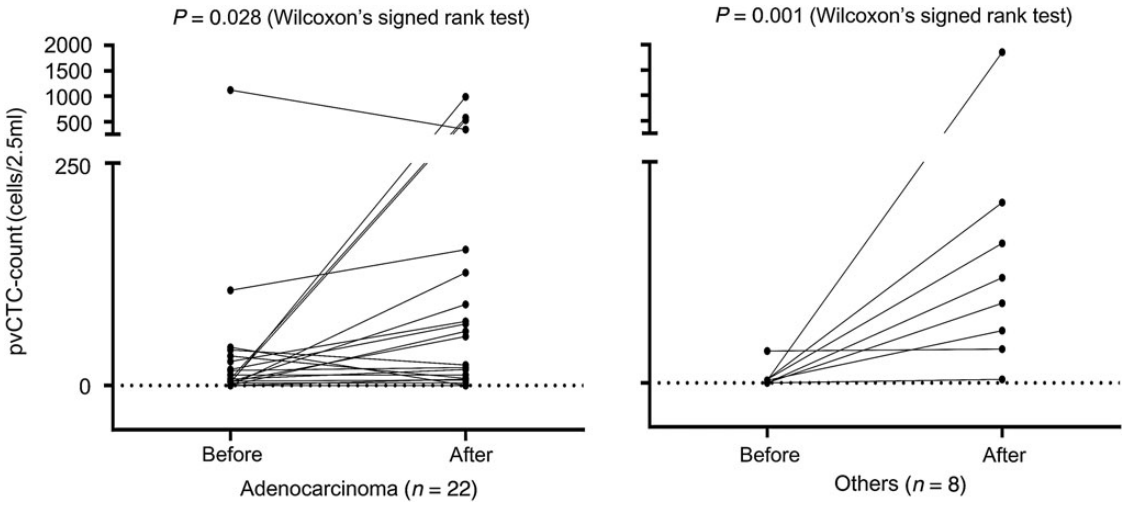

Figure 2: Comparison of the number of circulating tumour cells in 2.5 ml of drainage pulmonary venous blood (pvCTC-count) before and after surgical manipulation according to histology of primary tumour.

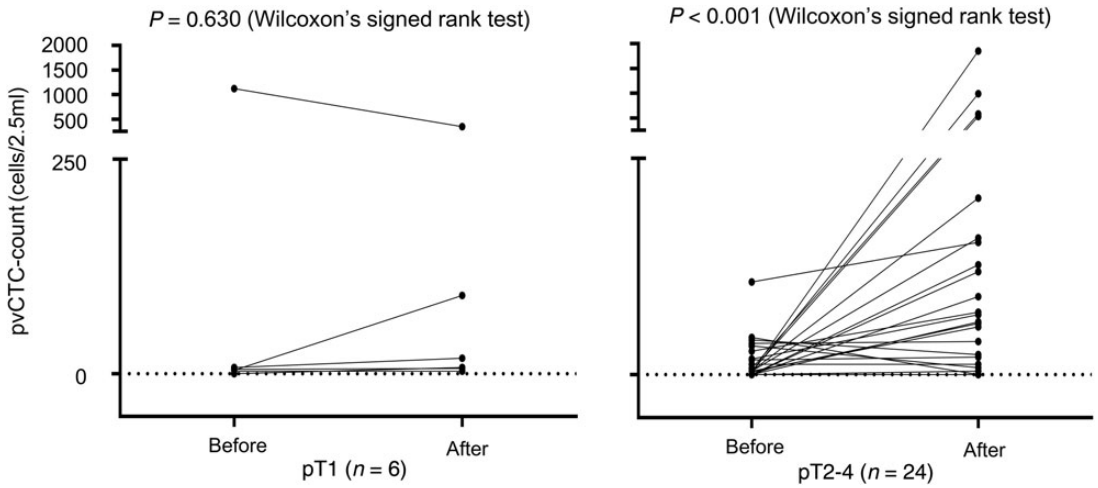

Figure 3: Comparison of the number of circulating tumour cells in 2.5 ml of drainage pulmonary venous blood (pvCTC-count) before and after surgical manipulation according to pathological tumour factor (pT).

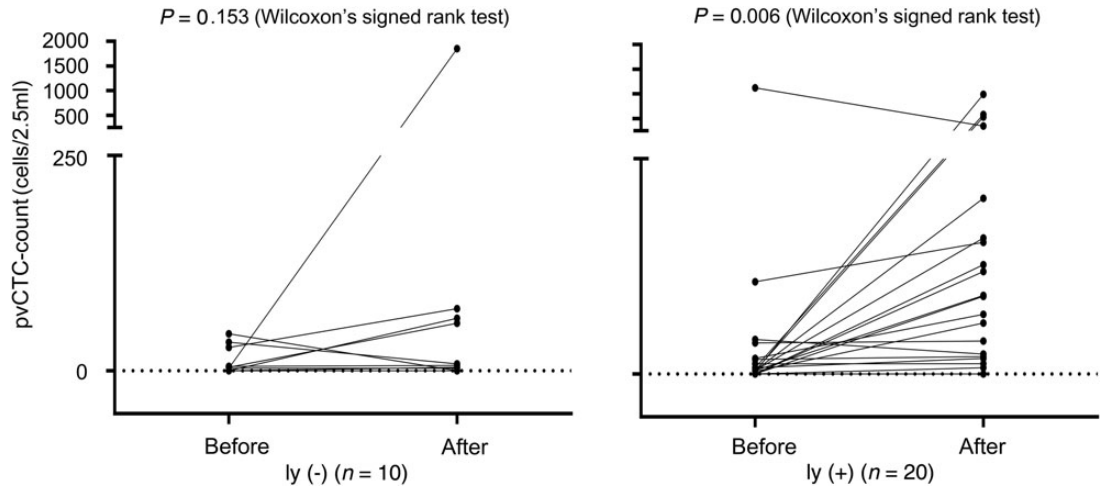

Figure 4: Comparison of the number of circulating tumour cells in 2.5 ml of drainage pulmonary venous blood (pvCTC-count) before and after surgical manipulation according to microscopic lymphatic invasion (ly) of primary tumour.

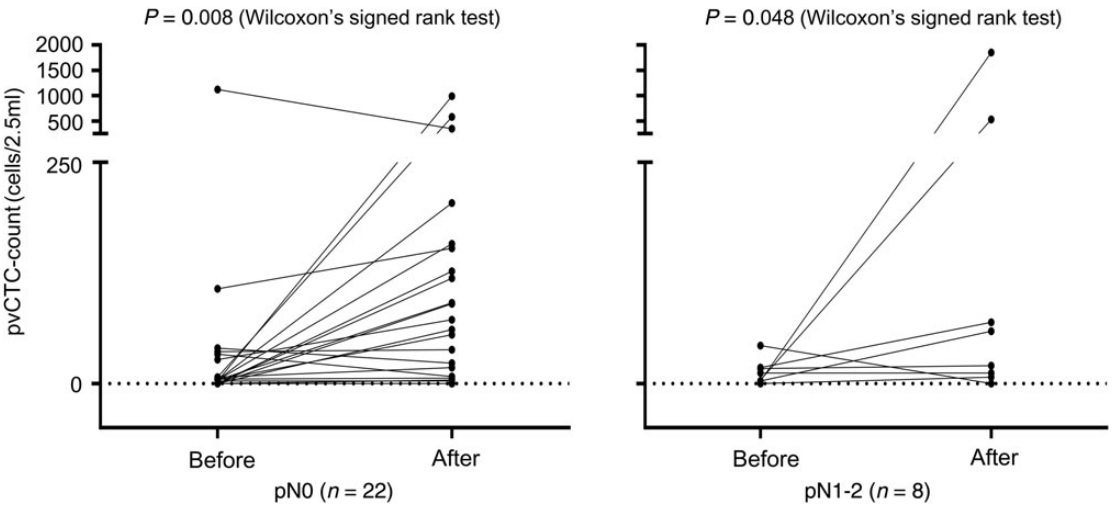

Figure 5: Comparison of the number of circulating tumour cells in 2.5 ml of drainage pulmonary venous blood (pvCTC-count) before and after surgical manipulation according to pathological nodal factor (pN).

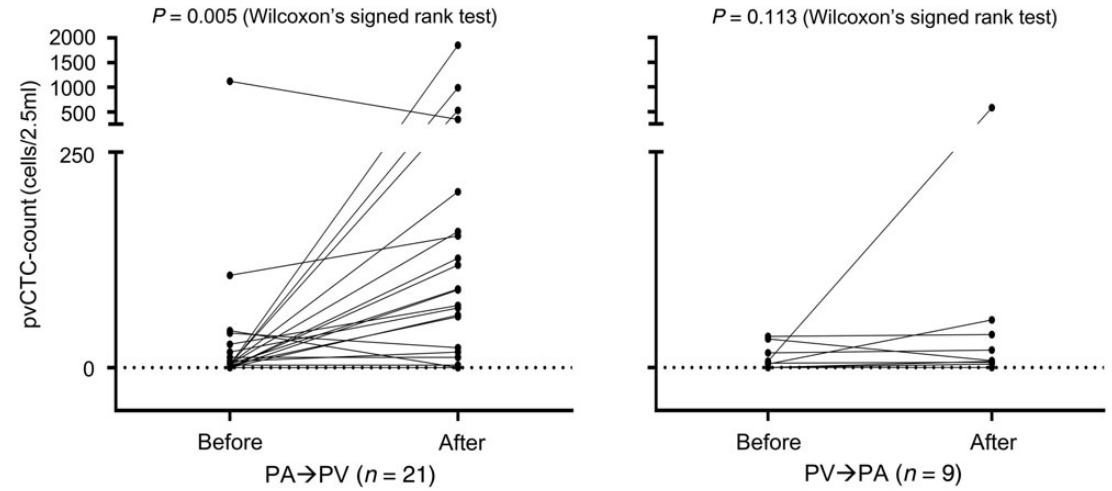

Figure 6: Comparison of the number of circulating tumour cells in 2.5 ml of drainage pulmonary venous blood (pvCTC-count) before and after surgical manipulation according to the sequence of vessel ligation, pulmonary artery (PA) ligation followed by pulmonary vein (PV) ligation (PA first) or vice versa (PV first).

established and may be partly revealed by long-term follow-up of the patients included in the study.

The most important result of the present study was a significant increase in the pcCTC-count after surgical manipulation (median pvCTC-count, per 2.5 ml of PV blood, 4.0 before manipulation and 60.0 after manipulation). The median number of CTCs after manipulation was similar to that documented in our previous study (81 cells/7.5 ml of PV blood) [11], which may suggest reproducibility of the CellSearch system in the quantitative evaluation of CTCs. A significant increase in the number of tumour cells in drainage PV blood during surgery for NSCLC was also observed in a study conducted by Song and coworkers [14]. In order to evaluate CTCs in PV blood they employed an indirect method, quantitative reverse transcriptase–polymerase chain reaction (RT-PCR), to evaluate gene expression of the tumour-specific antigens CK19 and CD44v6. They showed that the gene expression of CK19 and CD44v6 increased significantly after PA-first lobectomy, suggesting that surgical manipulation may produce spillage of tumour cells into the circulation through the drainage PV and seeding of micrometastases to distant organs.

In the present study, the pvCTC-count increased significantly in patients with tumours with positive lymphatic invasion, but not in those with tumours without lymphatic invasion. The exact reason for the difference in the increase in pvCTC-count according to the status of lymphatic invasion is unclear, and the results were obtained from a relatively small number of patients. Further studies are necessary to confirm the results and to reveal the reasons. The incidence of lymphatic invasion in the present study was 33.3%, which was somewhat higher than those reported in previous studies; the incidence in a recent large study was 20.6% (124 of 603) in pathological stage I disease [15]. However, as the incidence of lymphatic invasion varies in different studies even in pathological stage I disease (3.5–49.0% in a meta-analysis [16]), the incidence of lymphatic invasion documented in the present study (33.3%) seems not to be unusual.

In the present study, we examined CTCs in peripheral blood only before thoracotomy and did not assess changes in the number of CTCs resulting from surgical manipulation, which have been reported in some studies [17, 18]. Kurusu and coworkers examined CTCs with an indirect method, RT-PCR, to evaluate gene expression of a tumour-specific antigen (carcino-embryonic antigen, CEA), and showed that 64.3% (9 of 14) CEA-negative peripheral blood samples taken immediately before surgery turned into CEA-positive samples taken at the completion of lobectomy for NSCLC [17]. Sawabata and coworkers evaluated peripheral blood samples with the same detection system as employed in our study (the CellSearch system), and showed conversion from CTC-negative to CTC-positive immediately after thoracotomy in two of nine patients with c-stage I NSCLC [18]. Rolle and coworkers evaluated CTCs in peripheral blood samples taken before surgery with another direct detection system (the MAINTRAC system), and showed that CTCs were detected in 86% (20 of 23) patients with NSCLC [19]. Two weeks after surgery, CTCs remained positive in 20 initially CTC-positive patients, and CTCs turned from negative to positive in the other three patients [19]. These results may suggest that spillage of tumour cells from the primary tumour into the circulation can be induced by surgical manipulation. In fact, some studies have reported the development of distant metastases, probably caused by surgical manipulation during lung cancer surgery [20, 21].

Concerning the sequence of ligation of vessels, PA and PV, during lobectomy, to avoid possible spillage of tumour cells during lung resection, it might theoretically be recommended that the drainage PV is ligated first (PV first), especially in cases of tumours with higher risk of spillage [21, 22]. In fact, some randomized studies on changes in CTCs during lung resection, in which patients were randomly assigned to receive PV-first lobectomy or to receive PA-first lobectomy, have suggested an advantage of PV-first ligation during lobectomy for NSCLC [14, 17]. Kurusu and coworkers showed that positive conversion, i.e. from negative to positive, of tumour-specific gene (CEA) expression during surgery was more common in their PA-first lobectomy group than in their PV-first lobectomy group [85.7% (six of seven patients) and 42.9% (three of seven patients), respectively] [17]. Likewise, Song and coworkers reported that gene expression of tumour-specific genes (CK19 and CD44v6) significantly increased during surgery in their PA-first group but not in their PV-first group [14].

In spite of the possible advantage of the PV-first procedure to avoid spillage of tumour cells by surgical manipulation, the advantage has not been established clinically. Rafaely and coworkers conducted a prospective study on the effect of the sequence of vessel ligation during lobectomy for NSCLC, and showed no difference in tumour recurrence after surgery [23]. In the present study, we failed to show any difference in pvCTC-count after surgical manipulation, although patients were not randomly assigned into PV-first lobectomy or PA-first lobectomy groups. The results may support the suggestion that PV first provides no advantage over PA first in prevention of spillage of tumour cells by surgical manipulation.

It is possible PA first, principally employed in the present study, may have an advantage in immediate cessation of pulmonary blood supply, which may contribute to the prevention of unnecessary blood loss and/or pulmonary congestion. However, in a clinical study on the sequence of vessel ligation during lobectomy for NSCLC conducted by Yellin and coworkers, they failed to show any significant difference in blood loss during lobectomy or in the amount of blood retained in the resected lobe [24]. Accordingly, no advantage of PA first over PV first or of PV first over PA first has been established clinically, and the sequence can therefore be determined by the surgeon's preference. However, to avoid the risk of intraoperative systemic dissemination, it is recommended to avoid excessive manipulation of the tumour-bearing lobe, especially before ligation of the drainage PV, if venous invasion is suspected on preoperative computed tomography and/or magnetic resonance imaging.

In conclusion, we first showed direct evidence of a significant increase in tumour cells in the drainage PV during lobectomy for primary lung cancer, suggesting spillage of tumour cells caused by surgical manipulation. The clinical significance of the presence of tumour cells in PV blood and of the significant increase in tumour cells in PV blood during surgery should be assessed by long-term follow-up of patients included in the study. Furthermore, the importance of vessel ligation during lobectomy might be evaluated in future randomized studies.

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APPENDIX. CONFERENCE DISCUSSION

Dr G. Friedel *(Gerlingen, Germany)*: Although the problem of circulating tumour cells has existed for many years, until recently it was not in the main focus of research, and nowadays more and more importance is attached to it. There are many papers which could show the prognostic significance of circulating tumour cells. However, currently we do not have any idea how and at what time we should treat those tumour cells.

It seems quite plain that some surgical manipulations could be accountable for the spread of some of these cells. Our teachers must have already thought about this problem, and therefore we were instructed to perform the no-touch technique in oncologic surgery as well as to dissect and interrupt the pulmonary vein as the first step.

The main result of the present study is that patients with postoperative L1 lymphatic tumour invasion have more CTCs than L0 patients. This leads me to two questions. First, most of the patients in this study have early stage I and II, normally with a low proportion of patients with lymph node involvement. Therefore, the number of patients in your series with L1 postoperatively seems quite high to me. And the more complex question is: How do you explain this difference in significance? It should be a major part of your discussion. You didn't discuss this effect, and the most interesting result in your paper is that it is not the surgical technique that leads to the increased count of CTCs but the lymphatic spread. Could you comment on these two questions.

Dr F. Tanaka *(Kitakyushu, Japan)*: I am a co-author of this paper. Regarding the first question, the lymphatic invasion is checked microscopically. As you mentioned, the rate of lymphatic invasion is very high, but that is why there is a selection bias, because we directly sampled the PV blood from the drainage PV. It's a very risky procedure, and we included only patients who underwent open thoracotomy lobectomy alone. So very early stage patients operated on by a VATS procedure were excluded from this study. That's why the incidence of lymphatic invasion in the study might be higher.

Dr Friedel: Do you really think that the lymphatic spread is an effect of surgical technique? I agree that the increased count of circulating tumour cells could be an effect of the surgical technique but not the lymphatic spread, the perilymphatic spread.

Dr Tanaka: Your comment is right. The study showed a significant increase according to the lymphatic invasion, but the result was drawn from a very small number of patients included in this study. So I think a larger study should be conducted in the future.

Dr C. Sirois *(Montreal, Canada)*: Why did you puncture the pulmonary vein after the lobectomy? Why didn't you do it after the dissection but just before taking the pulmonary vein? Or maybe you could have done both?

Dr Tanaka: We conducted the study to assess the accumulation of circulating tumour cells in the drainage PV by manipulation of the lung during lobectomy. Your comment is correct. PV blood should be sampled from the drainage PV, not only after PV ligation as performed in the study, but also just before PV ligation to check circulating tumour cells as you commented.

Dr R. Milton *(Leeds, UK)*: I might have missed it, but did you look at the CTC count in peripheral blood after the lobectomy?

Dr Hashimoto: We didn't take it after the lobectomy.

Dr Tanaka: I think that's a very important point. But we actually failed to check the peripheral CTC after surgical manipulation, so again your comment is right.

Dr P. Rybojad *(Lublin, Poland)*: I'm interested, how did you choose the method? The method you used, is it a kind of flow cytometry or some other method?

Dr Hashimoto: The CellSearch system was the only method to capture tumour cells directly.

Dr Rybojad: So this is not a flow cytometry?

Dr Hashimoto: No.

Dr Rybojad: So how does it really work? Have you used antibodies or something else? How did you count the circulating tumour cells, how did you find them?

Dr Schmid: How did you identify the cells?

Dr Rybojad: I understand these are actual circulating tumour cells. As I understand right now there are two methods, flow cytometry or antibodies. So I wonder, what's your method?

Dr Tanaka: This question is how to check the –

Dr Schmid: How to identify: what's your method for identifying the circulating tumour cells with your system, what is the principle?

Dr Tanaka: Okay. In our system, circulating tumour cells were automatically captured with an antibody against an epithelial cell-specific antigen, EpCAM. So, strictly speaking, circulating tumour cells captured in our system means circulating epithelial cells. However, generally speaking, in normal conditions epithelial cells are not contained in the peripheral blood, so epithelial cells present in the peripheral blood roughly means circulating tumour cells.

Dr Rybojad: But in some cases, patients with lung cancer express the circulating tumour cells in the blood from the beginning of disease. You had some patients who had count zero. So I wonder how that was possible? What was your method? When you explained that it is epithelial cells, it makes me wonder even more how it happens that some patients didn't have circulating tumour cells at all at the starting point.

Dr Tanaka: Your comment is correct, and the low sensitivity of our system, the CellSearch system, to detect circulating tumour cells is the most critical issue. It is the only established system for clinical use and was approved (by the FDA in the USA) for breast cancer, colon cancer or prostate cancer, but not for lung cancer because of low sensitivity in identifying circulating tumour cells. Circulating tumour cells might be detected more frequently at an early stage with a more sensitive system.

Dr Rybojad: The lung tissue is very complex. It differs a lot from breast cancer and colon cancer. You have a lot of epithelial cells here, so it's very important to distinguish between normal epithelial cells or those which are really circulating tumour cells. But there are several methods right now, so I guess you should maybe use some other methods.

Dr Tanaka: Thank you for your important comment.

Dr Rybojad: One more brief question about lymph nodes. Did you see any differences between circulating tumour cells in relation to staging?

Dr Tanaka: In this study we demonstrated no significant change in circulating tumour cells according to clinical or pathological stage, as well as nodal status.

Dr Rybojad: Because in our country some researchers use a method with small IV catheters with antibodies and they found that the amount of circulating tumour cells was very much associated with staging and it really mattered if lymph nodes were involved or not. And the other thing which was found is that the manipulation is an important factor, but not the type of surgery. It doesn't matter if you perform lobectomy, pulmonectomy or segmentectomy. The amount of circulating tumour cells was dependent on the manipulation. So, open surgery produced more circulating tumour cells than the VATS technique. So there are a lot of things to study.

eComment. Circulating tumour cells caused by surgical manipulation in patients with lung cancer. Is minimally invasive "no-touch" surgery the solution?

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We read with great interest the article by Hashimoto *et al.* [1], reporting a significant increase in circulating tumour cells (CTCs) in pulmonary venous blood after surgical manipulation during lung cancer surgery. This increase seemed to be even more evident in patients with positive lymphatic invasion. In 73% of patients, CTCs were detected in peripheral blood samples beforehand. Whether these findings are correlated with long-term survival or increased risk of distant recurrence remain unanswered by the current study. The presence of CTCs before surgical resection and the subsequent increase after surgical manipulation might explain various interesting observations regarding long-term results after lung cancer surgery. First, a considerable number of lung cancer patients develop distant metastases after surgical treatment, especially with proven lymphovascular invasion, even after radical resection. It remains an interesting debate whether this is the result of preoperative presence of micro-metastases, undetectable by routine diagnostic modalities, or caused by intraoperative spillage of tumour cells with distant seeding of micro-metastases. Regardless, adjuvant chemotherapy has been proven to be beneficial for patients with stage II non-small cell lung cancer (NSCLC). CTC count may be an interesting biomarker to select patients with stage I NSCLC for adjuvant chemotherapy. Second, there is increasing evidence that long-term survival after NSCLC resection through video-assisted thoracic surgery (VATS) is at least equivalent to open surgery, with similar local control but possibly lower risk of systemic recurrence [2]. It has been suggested that such benefit after VATS may be attributed to reduced surgical trauma [2], leading to less immunosuppression [3] and better preservation of the patient's autologous tumour killing ability to clear micro-metastases or CTCs shed intraoperatively. In this regard, immunomodulation therapies offer great promise as a new adjuvant therapeutic strategy. Faster recovery associated with minimally invasive approaches might also lead to an earlier start of adjuvant chemotherapy when indicated. As such, we eagerly await future investigations assessing whether the introduction of robot-assisted techniques can further decrease surgical trauma of thoracic surgery and improve postoperative outcomes. How should this affect our views on surgical treatment of lung cancer patients? In colorectal cancer and pancreatic cancer surgery, the so- called "no-touch isolation technique" has long been advocated to reduce peroperative tumour cell dissemination, prioritizing on central vascular ligation before tumour mobilization [4–5]. However, further work is needed to determine the relevance of such techniques in lung cancer surgery. As stated by the authors of the current study, the sequence of vessel ligation during lung cancer resection might reduce the effects of increasing CTC count after surgical manipulation. Theoretically, the interruption of the draining pulmonary vein first (instead of ligation of the pulmonary arteries first) might avoid possible spillage of tumour cells in the circulation. Dividing the pulmonary vein first is already standard operative sequence in most VATS lobectomy procedures. This particular vessel ligation sequence, combined with decreased immunosuppression postoperatively, might be elements of the VATS approach contributing to its superior outcomes after lung cancer resection.

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