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ORIGINAL ARTICLE

Significance of tumour vessel invasion in determining the morphology of isolated tumour cells in the pulmonary vein in non-small-cell lung cancer

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Abstract

OBJECTIVES: The existence of clustered isolated tumour cells (ITCs) in the pulmonary vein (PV) of the lungs of patients with lung cancer has been reported to be a prognostic factor. However, the clinical–pathological characteristics related to their presence in the PV remain unclear.

METHODS: We analysed the surgical results and clinical–pathological findings of 130 patients who underwent surgery for non-small-cell lung cancer in regard to blood vessel invasion (BVI), serum carcinoembryonic antigen (CEA) level, maximum standardized uptake value (SUV-max), size of the solid region in computed tomography findings and pathological stage according to an ITC type, i.e. no tumour (N), singular tumour cells (S) and clustered tumour cells (C).

RESULTS: ITCs were detected in 96 (74%) of the patients, with C observed in 43, S in 53 and N in 34. Recurrence was seen in 33 (26%) cases, 21 of which were classified as C, 9 as S and 3 as N. The disease-free survival rate was significantly worse in C cases when compared with the others ($P < 0.01$). The rate of C was high in cases with high serum CEA, advanced p-staging and positive BVI ratio. Furthermore, BVI positive and ITC morphology were strongly related (BVI positive; 79 in C, 40 in S, 9% in N; $P < 0.01$).

CONCLUSIONS: Clustered ITCs were shown to be a prognostic indicator and strongly related to BVI. Our results suggest that determination of BVI has prognostic value, as clustered ITCs with metastatic potential are disseminated from the invaded vein.

Keywords: Blood vessel invasion • Isolated tumour cells • Surgery • Recurrence • Non-small-cell lung cancer

INTRODUCTION

Lung cancer is a leading cause of cancer death in most industrial countries [1]. In addition, an investigation of the causes of cancer deaths indicated that recurrence and distant metastasis occurred in approximately 70% of patients who underwent surgery [2]. Therefore, useful markers are needed for the early detection of distant metastasis and recurrence, with various prognostic biomarkers thus far reported. In blood chemical studies, serum carcinoembryonic antigen (CEA) has been shown to be one of the most useful tumour markers for providing information regarding cancer progression [3], while the presence of blood vessel invasion (BVI) in histopathological findings is also an important prognostic indicator [4, 5]. On the other hand, clinical imaging techniques such as computed tomography (CT) and positron emission tomography (PET) can also provide important information regarding cancer aggressiveness and malignancy. The solid lesion size of tumours in CT findings and maximum standardized uptake value (SUV-max) in PET imaging are helpful for clinical cancer treatment, as those factors are reported to reflect malignancy or metastatic potential [6, 7]. As for other biomarkers, the presence of isolated tumour cells (ITCs) in blood has been recently reported to be useful for determining prognosis, recurrence and metastasis [8–11]. In our previous report, we presented a novel method to enrich ITCs while maintaining their morphological appearance and then found relationships between ITC morphology and clinical backgrounds [12]. In cases with clustered ITCs, the recurrence rate was higher than that in cases with singular or no ITCs. From those results, we speculated that ITCs are shed from the primary tumour, then flow through a drainage vein and circulate throughout the whole body, easily leading to metastasis. In addition, relationships between ITCs and background factors of primary tumours were noted. In the present study, we assessed the relationships among clinical–pathological findings including BVI and morphological characteristics of ITCs in the pulmonary vein (PV) of resected lungs of non-small-cell lung cancer patients.

Table 1: Patient characteristics and distribution of ITCs in PV blood

	Total	PV cytology			*P*-value
		N	S	C	
Number	130	34	53	43	
Gender					
Male	74	22	26	26	0.8
Female	56	12	27	17	
Age in years (mean ± SD)	68 ± 9.0	67 ± 9.2	67 ± 9.3	69 ± 11.0	0.3
P-stage					
I	98	30	42	26	0.01
II	20	3	5	12	
III or IV	12	1	6	5	
Tumour histology					
Adenocarcinoma	92	21	41	30	0.8
Squamous cell carcinoma	26	8	10	8	
Miscellaneous	12	5	2	5	

PV: pulmonary vein; N: no tumour cells; S: singular tumour cells; C: clustered tumour cells.

MATERIALS AND METHODS

Detection of ITCs

The methods used for the detection of ITCs were reported in detail in our previous study [12]. Briefly, 50 µl/ml of a RosetteSep® Human CD45 Depletion Cocktail (Stemcell Technologies, Inc., Vancouver, Canada) was added to individual whole blood samples and mixed well. After incubation at room temperature, the mixture was diluted with an equal volume of phosphate buffered saline plus 2% fetal bovine serum and mixed gently. The diluted sample was then layered on the top of a Ficoll-Paque™ PLUS and centrifuged, then enriched ITCs were removed from the Ficoll-Paque™ PLUS–plasma interface. The cells were centrifuged down to polylysine-coated glass slides using a cytospin device. Cells on the slides were subjected to Papanicolaou staining.

Patients

One hundred and thirty consecutive patients (56 males, 74 females; range 28–88 years old, median 68.0 years) with primary non-small-cell lung cancer who did not undergo preoperative chemotherapy and/or radiation therapy were evaluated using our method (Table 1). Written informed consent was obtained from all enrolled patients. This study conformed to the ethical guidelines of Osaka University Graduate School of Medicine and was approved by the institutional review board of Osaka University Medical Hospital. All patients underwent a segmentectomy ($n = 11$), lobectomy or bilobectomy ($n = 119$) during systematic mediastinal lymphadenectomy procedures performed from August 2008 to 2010 at Osaka University Medical Hospital.

Postoperative staging of all patients was determined according to the tumour-node-metastasis (TNM) classification of the Union for International Cancer Control, ver. 7, 2009 (Table 1). The median follow-up duration was 19 (6–22 months). In follow-up examinations, all patients were evaluated at 3-month intervals. Each evaluation included a physical examination, chest X-ray and blood tests including tumour markers, while additional thoraco-abdominal CT scans were generally performed at 6-month intervals.

Blood samples, and ITC detection and enrichment

All blood samples were collected immediately after tumour resection by gently aspirating from the tumour-draining PV using an 18-gauge needle and placed in 10-ml ethylenediaminetetraacetic acid tubes. ITCs were isolated using a negative selection method from 1-ml blood samples using the method described in Section 2.1.

Evaluation and classification of clusters

Using all of the samples, one glass slide containing enriched ITCs from each patient was prepared and assessed by Papanicolaou staining. These examinations were performed independently by 2 cytologists (Eiichi Morii and Hideo Yoshimura) who were unaware of the patient's clinical data. For morphological assessment, each cytologist distinguished cancer cells from normal cells by light microscopy based on their morphological appearance, such as cell size and shape, nuclear size and shape and nuclear-cytoplasmic ratio. Furthermore, for cluster formation assessment, patterns of ITCs were classified into the following three types: no tumour cells (N), singular tumour cells (S) and clustered tumour cells (C). The correlations of patient characteristics with the distribution of ITC morphology in pulmonary venous blood are shown in Table 1. There were no significant differences regarding patient characteristics excluding p-staging among the three groups (N, S and C).

Clinical–pathological analyses according to morphological appearance

To reveal the relationships between clinical–pathological findings and ITC morphology, we examined the following parameters: serum CEA, size of solid primary tumour in CT findings, pathological stage and BVI evaluated by haematoxylin and eosin or elastic van Gieson staining. In patients with CEA ≥5 ng/ml, solid lesion size ≥20 mm in diameter and SUV-max ≥2.5 were considered to be elevated values [7, 13]. Statistical analysis was

performed using commercially available statistics software (JMP version 9, SAS Institute). A *P*-value <0.05 was considered to indicate a statistically significant difference. The characteristics of the patients were compared using the *t*-test, chi-square test and Fisher's exact test. Tukey–Kramer was used to compare the mean values of groups. For the analysis of follow-up data, survival curves were calculated using the Kaplan–Meier method, and survival distributions were compared with a log-rank test. In addition to examining the relationships among ITC morphology and clinical–pathological findings, we utilized logistic regression analysis. Furthermore, to examine the risk ratios of recurrence, a multivariate Cox's hazard model was used.

RESULTS

ITC classification and prognosis

ITCs classified as S were detected in 53 of the 130 patients (40%), while those classified as C were found in 43 (33%). During the median follow-up period of 19 months, 33 patients suffered cancer relapse, 21 in the C group, 9 in S and 3 in N. In the relapsed cases, exclusive local recurrence occurred in 11 (pleura in 7, chest wall in 2, hilar lymph node in 2, mediastinal lymph node in 1), local and distant metastases in 8 (bilateral lungs in 3, mediastinal lymph node and neck lymph node in 4, mediastinal lymph node and bone in 1) and exclusive distant metastasis in 13 (contralateral lung in 4, adrenal grand in 3, brain and liver in 2, other organs in 4). Relapse-free survival curves demonstrated that survival in the C group was significantly worse (Fig. 1). Table 2 also shows the 2-year recurrence free rate according to clinical parameters obtained during the follow-up period. From those results, all of the examined parameters showed an adequate predictive value.

Relationships of ITC morphology with clinical and histopathological findings

The relationships among ITC morphology, clinical background and histopathological findings are shown in Fig. 2. There were significant differences regarding ITC morphology for the clinical parameters serum CEA, positive BVI ratio and advanced p-stage. In cases classified as C, the levels of those parameters were elevated when compared with the N and S cases. In contrast, there were no significant differences regarding ITC morphology for elevated SUV-max and solid lesion size (Fig. 2). In addition, multivariate analysis revealed BVI to be one of the most important factors to determine the ITC morphology (Table 3).

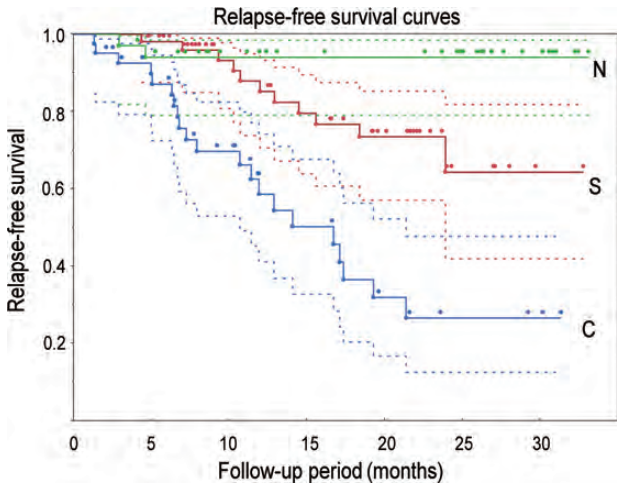

Figure 1: Relapse-free survival curves according to ITC morphology. N: no tumour cells; S: singular tumour cells; C: clustered tumour cells.

DISCUSSION

The present findings of blood samples taken from the PV of resected lungs of patients with lung cancer showed that the morphological pattern of ITCs was correlated with the clinical–pathological parameters for BVI. These results may provide new and important information regarding ITCs in the blood of affected patients.

The presence of ITCs in blood has been reported to be a useful biomarker in lung cancer patients [8–11]. We previously reported that ITCs in the PV were detected using a CD45-negative selection method and found that not only their presence but also cluster formation may be a prognostic indicator for early recurrence of lung cancer [12]. In cases with singular cancer cells, the recurrence rate was low when compared with those with cluster formation. However, it remains unclear whether ITC morphology is related to histopathological findings or clinical background. In the present study, we mainly investigated the relationships between the ITC morphological appearance and clinical pathological findings.

Recent studies have found that ITCs in blood have morphological and biological diversity. Hou *et al.* [14] used a filtration method (isolation by size of epithelial tumor cells [ISET]) to detect and enrich circulating tumour cells (CTCs) according to

Table 2: Relapse-free survival according to clinical–pathological parameters

	Variables	*N*	2Y-RFS (%)	*P*-value
1	ITCs in PV			<0.001
	None (N)	34	93	
	Singular (S)	53	64	
	Clustered (C)	44	26	
2	P-stage			<0.001
	Early (I)	98	71	
	Advanced (II, III, IV)	32	35	
3	Serum CEA			0.03
	Low (<5 ng/ml)	103	66	
	High (≥5 ng/ml)	25	43	
4	SUV-max			<0.001
	Low (<5)	51	77	
	High (≥5)	43	31	
5	BVI			<0.001
	Negative	71	92	
	Positive	59	30	
6	Solid lesion			<0.001
	<20 mm	81	87	
	≥20 mm	49	46	

2Y-RFS: 2-year relapse-free survival; ITCs: isolated tumour cells; PV: pulmonary vein; SUV: standardized uptake value; BVI: blood vessel invasion; Solid lesion: size of solid lesion in CT findings.

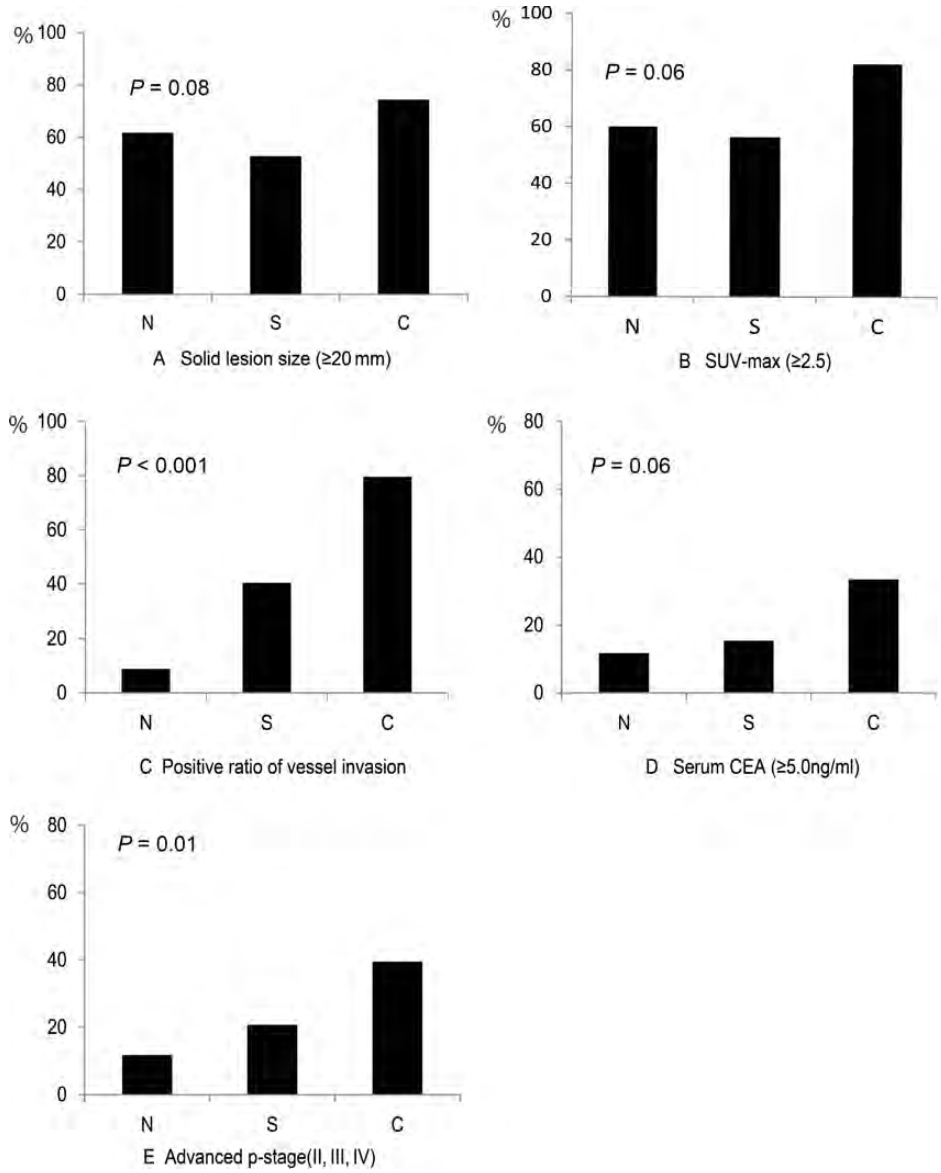

Figure 2: Relationships among ITC morphology and clinical parameters. The vertical axis indicates a high or positive ratio of each individual parameter. (**A**) Solid lesion size in CT findings (≥20 mm). (**B**). SUV-max in PET findings (≥5). (**C**) Blood vessel invasion. (**D**) Serum CEA (≥5 ng/ml). (**E**) Advanced pathological stage (II/III/IV).

the cell size obtained from the blood of lung cancer patients and then analysed their biological characteristics. Based on immunochemical staining results, they showed that clustered CTCs survive longer and have greater anti-apoptosis potential when compared with singular cells. Their findings correspond to our previous results.

As for other predictive factors, histopathological analyses have also provided important information about the biological characteristics of primary cancer. Table 2 shows the factors indicated to have a significant predictive value in the present study, with BVI found to be one of the most revealing prognostic biomarkers. Several groups have reported that BVI was a useful predictive factor for recurrence and metastasis [4, 5]. However, the related mechanisms have not been fully elucidated. In the present study, we found that the presence of clustered cancer cells was strongly related to BVI by primary tumours, as the positive ratio of BVI was significantly higher in cases with clustered ITCs when compared with the N and S groups. Our results showed that clustered ITCs in blood were seen in most cases with BVI, whereas singular cancer cells were also found in cases without BVI. This discrepancy may be because singular ITCs are more easily shed from microvascular vessels around the primary tumour that are too small to be diagnosed as positive BVI, while clustered ITCs may enter the bloodstream mainly as a result of large vessel invasion in an amount adequate for a BVI positive diagnosis. Our findings suggest that BVI indicates not only the presence of singular cancer cells in the bloodstream, but also clustered cancer cells with metastasis potential. In addition, clustered cancer cells may be easily shed from the invaded vein into the bloodstream in cases with vessel invasion, resulting in metastasis. For further analysis of the prognostic recurrent value of these clinical parameters, risk ratios after excluding apparent local recurrence cases were calculated using the Cox's proportional hazard model (Table 4). Those results also revealed BVI as

Table 3: Multivariate analysis of clinical–pathological parameters related to morphology of ITCs

	P-value	Odds ratio	95% CI
S/N			
BVI (+)	<0.01	5.85	1.54–30.04
CT (solid >2 cm)	0.25	0.49	0.13–1.65
CEA (>5 ng/ml)	0.31	1.56	0.33–8.68
P-stage (II, III, IV)	0.8	1.19	0.24–6.07
SUV-max (>2.5)	0.8	1.19	0.31–4.75
C/N			
BVI(+)	<0.01	103.38	15.3–2215
CT (solid >2 cm)	0.77	0.73	0.07–5.21
CEA (5 ng/ml)	0.39	2.33	0.34–19.75
P-stage (II, III, IV)	0.56	1.91	0.18–20.3
SUV-max (>2.5)	0.21	0.21	0.008–2.23

CI: confidence interval; N: no tumour cells; S: singular tumour cells; C: clustered tumour cells; BVI: blood vessel invasion; SUV: standardized uptake value.

Table 4: Multivariate analysis of the prognostic value in recurrent cases excluding local recurrence

	P-value	Risk ratio	95% CI
CEA (>5 ng/ml)	0.79	1.16	0.35–3.29
CT (solid >2 cm)	0.31	2.8	0.42–55.54
BVI	0.02	4.27	1.14–20.38
P-stage (II, III, IV)	0.04	2.98	1.01–8.93
SUV-max (>2.5)	0.8	3.54	0.88–24.71

CI: confidence interval; BVI: blood vessel invasion; SUV: standardized uptake value.

an independent predictive factor, while they suggest that the presence of BVI is an important factor to determine the morphology of ITCs in blood and the relationship to distant metastasis.

In conclusion, the presence of ITCs in the PV of resected lungs of lung cancer patients was shown to be related to varied morphologies, while significant relationships were found among clinical factors and histopathological findings. Importantly, BVI may indicate the presence of clustered ITCs. Our results support the notion that BVI is a predictive factor for early recurrence and distant metastasis, as a large number of clustered ITCs with metastatic potential may be disseminated from invaded blood vessels. These findings should be helpful to further elucidate the mechanism of metastasis in lung cancer.

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