ORIGINAL ARTICLE

Detection of circulating tumor cells using oHSV1-hTERT-GFP in lung cancer

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Abstract

Background: This study was conducted to evaluate the clinical utility of the oHSV1-hTERT-GFP circulating tumor cell (CTC) detection method in the peripheral blood of patients with lung cancer by comparing its sensitivity to the CellSearch CTC detection method.

Methods: The oHSV1-hTERT-GFP and CellSearch CTC detection methods were compared using peripheral blood samples of patients pathologically diagnosed with lung cancer.

Results: A total of 240 patients with lung cancer were recruited, including 89 patients who were newly diagnosed and 151 patients who had previously received treatment. Sixty-six newly diagnosed patients were evaluated using both methods. The CTC detection rates were 71.2% and 33.3% using the oHSV1-hTERT-GFP and CellSearch methods, respectively; this difference was statistically significant ($P = 0.000$). Among the entire cohort ($n = 240$), the CTC detection rate using the oHSV1-hTERT-GFP method was 76.3%, with a CTC count of 0–81. The CTC detection rates were 76.7%, 68.9%, and 76.3% in patients with squamous cell carcinoma, adenocarcinoma, and small cell lung cancer, respectively. There was no statistically significant difference in the CTC detection rates between these different pathological subtypes ($P = 0.738$). The CTC detection rates of 79.8% and 74.4% in patients with stage I–III and IV lung cancer, respectively, were not significantly different ($P = 0.427$).

Conclusion: The oHSV1-hTERT-GFP method is highly effective for detecting CTCs in patients with lung cancer, independent of pathological type and disease stage, and is ideal for large-scale clinical applications.

Introduction

The mortality rate of lung cancer, one of the highest globally, continues to increase. Current diagnostic approaches for patients with lung cancer rely mainly on imaging studies and pathologic assessment. A previous study reported that at the time of diagnosis, cancer was confined to the primary site in only 15% of cases, had spread to the lymph nodes or directly above the primary site in 22%, and more strikingly, metastatic cancer was found in 57% of cases.[1] Consequently, the five-year relative survival rates in these cases were 54%, 26.5%, and 4%, respectively. The main causes underlying the differences in survival rates were tumor recurrence and systemic metastasis.

Even after surgical treatment for early-stage lung cancer, 30% of patients experience recurrence or metastasis within five years. Furthermore, even patients diagnosed with an early-stage lung cancer based on current clinical staging criteria are reported to exhibit signs of distant metastasis.[2,3] Tumor cells that detach from the primary tumor can enter the blood and/lymph circulation via the blood and lymph vessels and are termed circulating tumor cells (CTCs); this comprises the first stage of local and distant metastases.[4] Unfortunately, conventional examination methods, such as imaging and pathological assessment, are rarely effective in the identification of these concealed micrometastases. Therefore, CTCs evidently play a

vital role in early diagnosis,[5–7] recurrence, and the monitoring of metastasis,[8,9] prognosis,[10] efficacy evaluation,[11] and individualized therapy of cancer patients.

As the number of CTCs is very small, with only about 1 CTC/10^6–10^7 white blood cells, the detection of CTCs using conventional methods remains a challenge, with consequent long-lasting limitations to research and applications. However, rapid development of CTC detection techniques has led to the emergence of increasingly feasible methods, such as CTC-Chip,[12–14] which is a microfluidic-based technology, subtraction enrichment and immunostaining-fluorescence in situ hybridization,[15] and targeted PCR for CTC.[16] Several studies on lung cancer have also assessed the utility of the CellSearch system.[10,17,18] In particular, Krebs *et al.* detected CTCs in peripheral blood samples of patients with non-small cell lung cancer (NSCLC), with a positive rate of 21% (more than 2 CTCs/ 2 CTCs per 7.5 mL peripheral blood).[10] Because the CTC detection rate is correlated with tumor stage, pathological type, and metastasis, and given that the number of CTCs can be utilized to assess the curative effect of therapies and prognosis, the number of CTCs is an effective factor predictive of progression-free and overall survival. However, CellSearch, which detects viable CTCs based on epithelial cellular adhesion molecule (EpCAM) expression, cannot detect CTCs with downregulated or deleted EpCAM. In addition, CellSearch cannot separate living cells for subsequent genetic testing and guidance in clinical decision-making. To overcome these limitations, we developed a new method for CTC detection based on telomerase-specific, replication-selective oncolytic HSV-1 that targets telomerase reverse transcriptase-positive cancer cells and expresses a green fluorescent protein that identifies CTCs (oHSV1-hTERT-GFP), with high sensitivity and specificity.[19,20] This method is independent of specific molecular structures on the tumor cell surface and can be used to isolate intact living cells for further analysis of tumor cell characteristics and molecular subtypes. In this prospective study, we evaluated the efficacy of the oHSV1-hTERT-GFP method for the detection of CTCs in lung cancer.

Methods

Patients

This study was conducted at the Affiliated Hospital of the Academy of Military Medical Sciences from September 2014 to January 2017. The inclusion criteria were as follows: pathological diagnosis of lung cancer, age ≥ 18 years, Eastern Cooperative Oncology Group performance status 0–2, and stage I–IV. In addition, only patients diagnosed with untreated squamous cell carcinoma, adenocarcinoma, or small cell carcinoma were assessed by both the oHSV1-

hTERT-GFP and CellSearch CTC detection methods, whereas those who were assessed only by the oHSV1-hTERT-GFP method were not limited to a specific pathological type or treatment history. Patients with a history of malignancy within the previous five years were excluded.

Sample collection

A 4 mL peripheral venous blood sample was collected into ethylenediaminetetraacetic acid-containing vacuum tubes from all patients and stored and transported at 2–8°C. All samples were processed within 4 hours in preparation for CTC detection using the oHSV1-hTERT-GFP method. For the detection of CTCs using the CellSearch method, 10 mL of peripheral venous blood from each patient was collected into a CellSave storage tube (Menarini Silicon Biosystems Inc., San Diego, CA, USA) which was stored and processed at room temperature, and samples were evaluated within 96 hours following blood collection.

oHSV1-hTERT-GFP circulating tumor cell (CTC) detection method

Circulating tumor cell detection using the oHSV1-hTERT-GFP method was achieved in two steps. First, the key transcriptional regulatory protein of HSV-1 was controlled by the hTERT promoter core sequence.[20] We then established a novel method to selectively label the CTCs in peripheral blood using GFP expression, which can be monitored by flow cytometry or fluorescence microscopy. Second, we isolated the CTCs using flow cytometry. In this study, the detection of ≥ 4 CTCs per 4 mL blood was defined as CTC-positive.

CellSearch CTC detection method

As previously reported, the CellSearch method includes the following steps.[21,22] First, 7.5 mL of blood was transferred from the CellSave tube into a centrifuge tube, and the sample was placed into the CellTracks AutoPrep system, which automatically concentrated and transferred the CTCs to the MagNest cell presentation device for incubation with light exposure (Immunicon Corporation, Huntingdon Valley, CA, USA). The presence of CTCs was then determined using the CellTracks Analyzer (Menarini Silicon Biosystems Inc.), which automatically scans for fluorescent signals from the MagNest samples. Finally, the data on CTCs were recorded and interpreted manually.

Statistical analysis

SPSS version 19 (IBM Corp., Armonk, NY, USA) was used to analyze data and calculate numbers, means, medians,

and minimum and maximum values for quantitative data and to determine numbers, rates, and percentages for qualitative data. For the analysis of quantitative data that satisfied both normality and homogeneity of variance, a Student's *t*-test was used, whereas Wilcoxon's rank-sum test was used for data not satisfying normality or homogeneity of variance. For enumeration data, a chi-squared or Fisher's exact probability test was used in accordance with the specific circumstances of data. To assess correlations between CTC number and various variables, Spearman's rank-order correlation analysis was used. The level of significance was set to 0.05 for all tests performed.

Results

Demographic characteristics of patients

A total of 240 patients with lung cancer who fulfilled the inclusion criteria were included in the study: 138 men (57.5%) and 93 women (42.5%) (Table 1). The median age was 58 years (range 31–87). Two hundred and two (84.2%)

patients had NSCLC, including squamous cell carcinoma (*n* = 30), adenocarcinoma (*n* = 167), and others (adenosquamous carcinoma, *n* = 4; neuroendocrine carcinoma, *n* = 1). Stage I–II, III, and IV disease was detected in 35, 31, and 136 patients, respectively. There were 38 (15.8%) patients with small cell lung cancer (SCLC), including 18 with limited disease (LD) and 20 with extensive disease (ED). In this study cohort, 127 patients (52.9%) were non-smokers, 22 (9.2%) smoked < 400 cigarettes per year, and 91 (37.9%) smoked ≥ 400 cigarettes per year. Finally, 89 patients (37.1%) were newly diagnosed (Table 2), whereas the remaining 151 patients (62.9%) had been previously treated (Table 1).

Comparison of oHSV1-hTERT-GFP and CellSearch CTC detection methods

We compared the oHSV1-hTERT-GFP and CellSearch CTC detection methods in 66 of the 89 patients newly diagnosed with lung cancer. The CTC detection rates and CTC counts were 71.2% (47/66) and 33.3% (22/66), and 0–59 and 0–903 using the oHSV1-hTERT-GFP and CellSearch methods, respectively (Table 3). These results indicated that a significantly higher number of CTCs were

Table 1 Baseline characteristics of lung cancer patients

Variables	Patients (*n* = 240)	
	No.	%
Age (years)		
Median age	58	
Range	31–87	
Gender		
Male	138	57.5
Female	102	42.5
Smoking status (per year)		
0	127	52.9
< 400	22	9.2
> 400	91	37.9
Pathological type		
Squamous cell carcinoma	30	12.5
Adenocarcinoma	167	69.6
Small cell carcinoma	38	15.8
Others	5	2.1
Disease stage		
NSCLC (*n* = 202)		
I–II	35	14.6
III	31	12.9
IV	136	56.7
SCLC (*n* = 38)		
LD	18	7.5
ED	20	8.3
Treatment history†		
Yes	89	37.1
No	151	62.9

†Treatment history: anti-tumor treatment, including radiotherapy, chemotherapy, targeted therapy, and immunotherapy. ED, extensive disease; LD, limited disease; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

Table 2 Baseline characteristics of patients newly diagnosed with lung cancer

Variables	Patients (*n* = 66)	
	No.	%
Age (years)		
Median age	59	
Range	37–87	
Gender		
Male	38	57.6
Female	28	42.4
Smoking status (per year)		
0	33	50.0
< 400	4	6.1
> 400	29	43.9
Pathological type		
Squamous cell carcinoma	10	15.2
Adenocarcinoma	45	68.2
Small cell carcinoma	11	16.7
Disease stage		
NSCLC (*n* = 55)		
I	5	9.1
II	6	10.9
III	6	10.9
IV	38	69.1
SCLC (*n* = 11)		
LD	6	54.5
ED	5	45.5

ED, extensive disease; LD, limited disease; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

Table 3 The results of oHSV1-hTERT-GFP and CellSearch CTC detection methods

	CellSearch		
oHSV1-hTERT-GFP	+	−	Total
+	22	25	47
−	0	19	19
Total	22	44	66

CellSearch: circulating tumor cells (CTCs) ≥ 1/7.5 mL, the CTC detection rates and CTC counts were 33.3% (22/66) and 0–903, respectively. oHSV1-hTERT-GFP: CTCs ≥ 4/4 mL, the CTC detection rates and CTC counts were 71.2% (47/66) and 0–59, respectively.

detected using the oHSV1-hTERT-GFP method than the CellSearch method ($P = 0.000$). All patients determined as CTC-positive by the CellSearch method were also determined as CTC-positive by the oHSV1-hTERT-GFP method (22/22, 100%). In contrast, of the 47 patients determined as CTC-positive using the oHSV1-hTERT-GFP method, only 22 patients (46.8%) were subsequently determined as CTC-positive using the CellSearch method. Furthermore, in patients with SCLC and advanced lung adenocarcinoma, the rates of CTC detection using the oHSV1-hTERT-GFP method were significantly higher than the CellSearch method ($P = 0.031$ and $P = 0.000$, respectively). Table 4 summarizes the CTC detection rates using the two detection methods in patients with different pathological types and clinical stages.

CTC detection by oHSV1-hTERT-GFP

As presented in Tables 5 and 6, we next evaluated the utility of the oHSV1-hTERT-GFP CTC detection method in 240 patients with lung cancer, which revealed a detection rate of 76.3% (183/240), and a median CTC count of seven (range 0–81). In the 240 patients studied, the CTC detection rates were 80% (24/30), 74.9% (125/167), and 76.3% (29/38), and the CTC count ranges were 0–43, 0–81, and 0–28, for squamous cell carcinoma, adenocarcinoma, and

SCLC patients, respectively. In the remaining five patients with other lung cancer types (2.1%), the CTC detection rate was 100% (5/5) and the CTC count range was 4–33. There were no significant differences in CTC detection rates between the different pathological types of lung cancer in this cohort ($P = 0.738$).

Further analysis revealed that the CTC detection rates in patients with stage I–III and IV lung cancer were 79.8% and 74.4%, respectively, which were not significantly different ($P = 0.427$). Only one of the 240 patients with lung cancer had neuroendocrine carcinoma with a CTC count of 33, and four patients with adenosquamous carcinoma had CTC counts of 10, 9, 4, and 6. The CTC detection rates in non-smoking patients, patients who smoked > 400 and patients who smoked ≥ 400 cigarettes per year were 76.7%, 68.9%, and 76.3%, respectively, which were not significantly different ($P = 0.761$).

Discussion

To the best of our knowledge, this is the first study to compare the oHSV1-hTERT-GFP and CellSearch CTC detection methods using the peripheral blood of patients with lung cancer in order to provide evidence that both methods are effective for the detection of CTCs in patients with stage I–IV lung cancer. However, intriguingly, the CTC detection rate using the oHSV1-hTERT-GFP method was significantly higher than when using the CellSearch method ($P = 0.000$). Furthermore, while 22 newly diagnosed patients (33.3% cases) were determined as CTC-positive by both the CellSearch and oHSV1-hTERT-GFP methods, an additional 25 (37.9%) patients diagnosed as CTC-negative using the CellSearch method were diagnosed as CTC-positive using the oHSV1-hTERT-GFP method. This observed advantage of the oHSV1-hTERT-GFP method over CellSearch may stem from the different approach used for CTC detection.[19] In addition, our

Table 4 Correlation analysis of oHSV1-hTERT-GFP and CellSearch CTC detection methods

Type	Stage	Total number of patients	oHSV1-hTERT-GFP		CellSearch		P
			Number of patients with ≥ 4 CTCs/4 mL blood	Sensitivity	Number of patients with ≥ 1 CTC	Sensitivity	
Adenocarcinoma	I–III	11	8	0.727	3	0.273	0.063
	IV	34	23	0.676	14	0.412	0.004
	Total	45	31	0.689	17	0.378	0.000
Squamous cell carcinoma	I–III	6	4	0.667	1	0.167	0.250
	IV	4	2	0.500	0	0	NA
	Total	10	6	0.600	1	0.100	0.063
Small cell lung cancer	LD	6	5	0.833	2	0.333	0.250
	ED	5	5	1.000	2	0.400	NA
	Total	11	10	0.909	4	0.364	0.031

CTC, circulating tumor cells; ED, extensive disease; LD, limited disease; NA, not available.

Table 5 CTC detection rates according to various characteristics of the study cohort

	CTC-positive number	
Characteristic	Number	%
Pathologic type		
Squamous cell carcinoma (*n* = 30)	24	80.0
Adenocarcinoma (*n* = 167)	125	74.9
Small cell carcinoma (*n* = 38)	29	76.3
Other (*n* = 5)	5	100
P	0.738	
Disease stage		
I–III (*n* = 84)	67	79.8
IV (*n* = 156)	116	74.4
P	0.427	
Smoking		
No (*n* = 127)	95	74.8
< 400/year (*n* = 22)	18	81.8
≥ 400/year (*n* = 91)	70	76.9
P	0.761	

CTC, circulating tumor cells.

findings indicated that the CTC detection rate using the oHSV1-hTERT-GFP method was higher than the Cellsearch method in patients with lung adenocarcinoma, squamous cell carcinoma, and small cell carcinoma. Similar results were observed in patients at different disease stages.

Among a total of 240 lung cancer patients, the CTC detection rate was 76.3% (183/240) using the oHSV1-hTERT-GFP method, which was consistent with the findings of our previous studies using the same method[19,20] and significantly higher than that reported by Krebs *et al.* using the CellSearch method (21%).[10] Many previous studies have analyzed the relationship between CTCs and different pathological types of lung cancer using the CellSearch method. For example, Fumihiro *et al.* reported that CTCs were associated with the histologic type of lung cancer and that the number of CTCs in SCLC was higher than in NSCLC.[23] Yoshitomo *et al.* determined that the CTC detection rate was distinctively higher in lung squamous cell carcinoma than in lung adenocarcinoma (*P* = 0.028).[18] Furthermore, Krebs *et al.* reported a significantly higher CTC detection rate in lung adenocarcinoma than in lung squamous cell carcinoma patients (*P* = 0.013).[10] Our study revealed that the CTC detection rate using the oHSV1-hTERT-GFP method was not associated with pathological types, which was consistent with the results of a study by Hofman *et al.*[17]

Findings from many studies have also indicated that the presence of CTCs is closely associated with tumor stage and that the detection of CTCs in peripheral blood is indicative of metastatic status within tumor node metastasis staging.[24] CTC counts in the peripheral blood of patients with advanced lung cancer and distant metastases are significantly higher than in patients with early-stage cancer.[10,23,25,26] CTCs detected in patients with early-stage

Table 6 CTC detection rates by oHSV1-hTERT-GFP method in lung cancer patients

Type	Stage	Total number of patients	Number of patients with ≥ 4 CTCs	Median CTCs (range)	Mean CTCs	Sensitivity
Adenocarcinoma	I/II	27	22	6 (1–37)	7.81	0.815
	IIIa	10	7	6 (0–12)	5.90	0.700
	IIIb	8	5	6.5 (1–28)	10.13	0.625
	IV	122	91	8 (0–81)	10.98	0.664
	Total	167	125	8 (0–81)	10.12	0.689
Squamous cell carcinoma	I/II	8	8	14 (8–26)	14.63	1.000
	IIIa	8	7	6.5 (1–22)	9.25	0.825
	IIIb	4	4	23.5 (5–43)	23.75	1.000
	IV	10	5	3.5 (0–25)	5.8	0.400
	Total	30	24	8.5 (0–43)	11.47	0.767
Small cell lung cancer	LD	18	13	4.5 (1–26)	6.78	0.722
	ED	20	16	6 (0–28)	9.35	0.800
	Total	38	29	5 (0–28)	8.13	0.763
Other NSCLCs	IIIa	1	1	/	/	1.000
	IV	4	4	/	/	1.000
	Total	5	5	/	/	1.000
Smoking	No	127	95	7 (0–81)	8.89	0.748
	< 400/year	22	18	7.5 (1–54)	12.27	0.818
	≥ 400/year	91	70	9 (0–59)	11.05	0.769
Total		240	183	7 (0–81)	10.02	0.7625

CTC, circulating tumor cells; ED, extensive disease; LD, limited disease; NSCLCs, non-small cell lung cancers.

lung cancer often indicate metastasis.[25,27] We found no significant differences in CTC detection rates or CTC count in patients with lung cancer at different disease stages, which further implied that the oHSV1-hTERT-GFP method may be able to detect CTCs independently of disease stage in lung cancer patients.

In conclusion, the current study provides evidence of the sensitivity of both the oHSV1-hTERT-GFP and CellSearch methods for CTC detection in the peripheral blood of patients with lung cancer. The clinical application of the oHSV1-hTERT-GFP CTC detection method in patients with lung cancer has been validated. Overall, these findings reveal high sensitivity of the oHSV1-hTERT-GFP CTC detection method for lung cancer, independent of the pathological type and disease stage. Furthermore, this method, which requires only a small sample volume (4 mL) and is easy to perform at a relatively low cost, is ideal for large-scale clinical applications.

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Disclosure

No authors report any conflict of interest.

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