Detection of *CK19*, *LUNX*, and *KS1/4* mRNA expression in the peripheral blood for diagnosis of micrometastases in patients with non-small cell lung cancer and their clinical implications

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ABSTRACT. The expression of *CK19*, *LUNX*, and *KS1/4* mRNA biomarkers was detected in the peripheral blood of non-small cell lung cancer (NSCLC) patients to investigate the feasibility of indicating lung cancer micrometastases. Micrometastases were identified in the peripheral blood of 32 NSCLC patients, 15 benign pulmonary disease (BPD) patients, and 10 healthy volunteers by reverse transcriptase-polymerase chain reaction. The detection rates of *CK19*, *LUNX*, and *KS1/4* mRNA-positive cells in the peripheral blood obtained from the NSCLC group were 34.4% (11/32), 37.5% (12/32), and 25% (8/32), respectively. *CK19*, *LUNX*, and *KS1/4* mRNA-positive cells were detected in 6.6% (1/15), 0.0% (0/15), and 13.3% (2/15) of the patients with BPD, respectively. However, the healthy group did not express any of the three markers. The expression of *CK19*, *LUNX*, and KS1/4 mRNA was significantly higher in the NSCLC group than that in the healthy and BPD groups (P < 0.05). *CK19* and *LUNX* mRNA may be ideal biomarkers indicating micrometastases in patients with NSCLC; however,

the diagnostic applicability of *KS1/4* mRNA remains uncertain. The rate of expression of *CK19* was not correlated with the clinicopathological characteristics ($P > 0.05$). The rate of expression of *LUNX* and *KS1/4* was closely related to the clinical stage ($P < 0.05$), and not related to the clinical characteristics of the disease (age, gender, smoking history, pathological type, histologic classification, and differentiation; $P > 0.05$).

Key words: Non-small cell lung cancer; Micrometastases; *CK19* mRNA; *LUNX* mRNA; *KS1/4* mRNA; RT-PCR

INTRODUCTION

Non-small-cell lung cancer (NSCLC) accounts for approximately 80% of the lung cancers (Peters et al., 2012). Surgery is the most common type of treatment for this type of cancer; however, the patients amenable to radical surgery have a 5-year overall survival rate of 50%. NSCLC recurrence or metastasis may be a result of residual cancer cells in the peripheral blood. The identification of an ideal biomarker to indicate micrometastases, and the development of effective intervention strategies helps improve the prognosis of NSCLC patients. The application of reverse transcriptase polymerase chain reaction (RT-PCR) for the detection of micrometastases in the peripheral blood of NSCLC patients offers a readily accessible biological parameter that may be useful in monitoring disease progression. In order to establish the value of biomarker-based surveillance of micrometastases in NSCLC patients, it is necessary to correlate the presence of NSCLC cells (detected by RT-PCR) with established clinicopathological indicators.

In this study, RT-PCR was used to detect the expression of *CK19*, *LUNX*, and *KS1/4* mRNA in the peripheral blood of NSCLC patients, and the correlation between these three mRNA-based biomarkers and the clinicopathological parameters of NSCLC patients was determined.

MATERIAL AND METHODS

Subjects

The control population consisted of 15 healthy volunteers with no clinical history of malignancy and 15 patients with benign pulmonary diseases (BPD). All patients included in the study had been newly diagnosed at our institution from February to November 2014. All patients underwent radical lobectomy and systematic lymph node dissection, but specifically denied any previous history of radiation or/and chemotherapy. The NSCLC group consisted of 32 patients (27 men, 5 women), aged between 45 and 70 years (mean age 58.9 ± 7.1 years). The study population consisted of 7 patients with low-differentiative NSCLC, 21 patients with moderately-differentiative NSCLC, and 4 patients with high-differentiative NSCLC. The lung cancer staging was revised based on the guidelines of the International Association for the Study of Lung Cancer (IASLC) system (Goldstraw and Crowley, 2006). Based on these criteria, 12, 11 and, 9 patients were classified into tumor stage I, II, and III, respectively.

Collection of venous blood samples

The study protocols were reviewed and approved by the institutional review board.

Peripheral blood samples were obtained from all patients and controls with informed consent. To prevent contamination, only the second sequential 5 mL blood sample collected in an ethylenediamine-tetra acetic acid (EDTA)-containing tube was used for analysis. The blood samples were transported on ice to the laboratory for RNA isolation within 2 h of collection.

Extraction of total RNA and reverse transcriptase PCR

Total RNA was isolated from 1 mL of the blood sample using RNAiso Blood (TaKaRa, Otsu, Japan) according to the manufacturer protocols. All RNA extracts were analyzed by agarose gel electrophoresis and UV spectrophotometry. First strand cDNA synthesis was performed using the PrimeScript™ RT Master Mix (Takara, Japan), according to protocols provided by the manufacturer. Specific *CK19*, *LUNX*, and *KS1/4* cDNA sequences were amplified using the TaKaRa Taq™ Version 2.0 plus dye (TaKaRa) according to the manufacturer protocol. In brief, a 25 µL reaction mixture, composed of 4 µL cDNA product (equivalent to 200 ng RNA), 12.5 µL 2X Taq PCR Mix, 1 µL specific primers (10 mM), and 6.5 µL RNA free H_2O, was used. The PCR was performed in aTProfessiona thermal cycler (Biometra, Germany), using the default cycling conditions (30 cycles of 95°C for 5s, 60°C for 30s, and 72°C for 30s, after an initial incubation at 95°C for 30s). Primers for 3 candidate genes were designed using the Primer Premier v 5.0 software program. The primer sequences are listed in Table 1. Expression values were normalized to that of the reference gene (*GAPDH*) gene. The experiments were repeated thrice, with 2 technical replicates for each experiment (Table 1).

Table 1. Real time polymerase chain reaction (RT-PCR) primers.

Gene	Primer sequence
CK19	Forward: 5'-AGGTGGATTCCGCTCCGGGCA-3'
	Reverse: 5'-ATCTTCCTGTCCCTCGAGCA-3'
LUNX	Forward: 5'-CTCATTGTCTTCTACGGGCTGTTAG-3'
	Reverse: 5'-CTTTATGCCGAGAGGGATGGT-3'
KS1/4	Forward: 5'-CGCAGCTCAGGAAGAATGTG-3'
	Reverse: 5'-TGAAGTACACTGGCATTGACGA-3'
GAPDH	Forward: 5'-CGGAGTCAACGGATTGGTCGTCGTAT-3'
	Reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

Statistical analysis

Clinical and pathological information was obtained from the clinical records of the patient. The relationship between the clinical and pathological parameters and the expression of the three mRNA-based biomarkers was initially explored by one-way analysis of variance (ANOVA), followed by the least significant difference multiple comparison test. All statistical analyses were performed on the SPSS (v.13.0) software platform for Windows (SPSS Statistics, IBM, Armonk, NY, USA). P values <0.05 were considered to be statistically significant.

RESULTS

Calibration of *CK19*, *LUNX* and *KS1/4* mRNA detection by RT-PCR

The expression of *CK19* mRNA, *LUNX* mRNA and *KS1/4* mRNA was initially examined by RT-PCR in the peripheral blood of NSCLC, BPD, and healthy groups. *CK19*, *LUNX*, and *KS1/4*

mRNA was detected 34.4% (11/32), 37.5% (12/32), and 25% (8/32) of the peripheral blood samples extracted from 32 patients with NSCLC, respectively. The positive expression rate of *CK19*, *LUNX*, and *KS1/4* mRNA was 6.6% (1/15), 0.0% (0/15), and 13.3% (2/15), respectively, in the BPD group, while the positive expression rate of the three mRNA-based biomarkers was 0.0% in the healthy group. The positive expression rate of *CK19*, *LUNX*, and *KS1/4* was found to be significantly higher in the NSCLC group than that in the BPD group ($P < 0.05$).

Association between clinicopathological parameters and detection of *CK19*, *LUNX*, and *KS1/4* mRNA in peripheral blood

The correlation between the detection of *CK19*, *LUNX*, and *KS1/4* mRNA in peripheral blood and the clinicopathological parameters of NSCLC patients is presented in Table 2. *LUNX* and *KS1/4* mRNA expression was closely related to the clinical stage of the disease; however, the mRNA expression of these two factors showed no correlation with the age, sex, smoking history, pathological type, histologic classification, and differentiation. On the other hand, *CK19* mRNA expression was also not significantly correlated with the age, sex, smoking history, pathological type, histologic classification, differentiation, and clinical stage of the disease (Table 2).

Table 2. The relationship between CK19, LUNX, or KS1/4 positivity, and clinicopathological characteristics in non-small-cell lung cancer (NSCLC).

Characteristics	Number	CK19 +	P value	LUNX +	P value	KS1/4+	P value
Age			0.390		0.647		1.000
≤50	6	3		6		1	
>50	26	8		26		7	
Gender			0.428		0.626		0.578
Male	27	10		27		6	
Female	5	1		5		2	
Smoking history			0.465		0.452		0.433
Yes	20	8		9		4	
No	12	3		3		4	
Primary tumor			0.703		0.465		1.000
T1	11	3		3		3	
T2	21	8		9		5	
Histological classification			0.425		0.248		0.681
Adenocarcinoma	10	2		2		3	
Squamous carcinoma	22	9		10		5	
Differential			0.374		0.374		0.646
Poor	7	1		1		1	
Moderate + high	25	10		10		7	
Stage			0.681		0.049		0.001
I + II	23	7		6		3	
III	9	4		6		7	

DISCUSSION

Micrometastases refers to the process with which non-haematological malignant cells spread and survive in the circulatory and lymphatic systems, bone marrow, and other organs, prior to the formation of metastatic nodules. Meanwhile, the development of micrometastases has no clinical manifestations, and it is therefore difficult to detect a single cell or cell colony using routine experimental methods (Coello et al., 2004). Ashworth discovered micrometastases in peripheral blood for the first time in 1869. Cancer cells enter the circulatory system; initially, a majority of the

cancer cells are cleaned by the body's immune system when transferred into the circulatory system. As a result of this process, only a few cancer cells are kept alive; however, these cells find it difficult to metastasize (Ono et al., 2009). Micrometastases need not metastasize. However, if cancer cells are detected in the peripheral blood, it is likely to metastasize and recur. Previous studies have utilized members of the cytokeratin family (CKS) as biomarkers of micrometastases. CK19 is specifically expressed in epithelial tumor and normal epithelial cells. The presence ofCK19-positive cells in peripheral blood suggests the existence of cancer cells (Arrieta et al., 2014). In our study, *CK19* mRNA was detected in 34.4% (11/32) of the peripheral blood samples obtained from NSCLC patients. This result suggested the transfer of cancer cells to the blood. However, false-positive cases have also been reported by some researchers. This may be related to contamination with skin cells at venipuncture (Dingemans et al., 1997). Strict precautions were employed in this study, and 1 mL original blood was discarded to avoid contamination with skin cells at venipuncture, in order to eliminate false-positive results. However, one *CK19*-positive patient was still found in the BPD group. This result may be attributed to tainted samples coming from inflammatory epithelial cells.

A previous report by Mitas et al identified *LUNX* to be a sensitive biomarker of lung cancer micrometastases (by RT-PCR), compared to five other cancer-associated genes (*muc1*, *KS1/4*, *CEA*, *CK19*, and *PSE*) (Mitas et al., 2003b). *LUNX* has also been proved to be the most sensitive biomarker, with a high expression in hydrothorax and peripheral blood (Cheng et al., 2008). In our study, we observed that 37.5% (12/32) of the NSCLC patients displayed a positive expression of *LUNX* mRNA in the peripheral blood, which was higher than that of the other two mRNA. However, the expression of *LUNX* mRNA was not tested in healthy and BPD groups. Further study revealed that the biomarker *LUNX* was closely related to the clinical stage. Therefore, it may be a useful indicator for predicting micrometastases in NSCLC patients.

Wallace et al. (2005) analyzed the expression of *LUNX*, *KS1/4*, *MUCI*, *CK19*, and *CEA* mRNA in the lymphaden of NSCLC patients to indicate micrometastases; they concluded that 93 and 56% of the patients displaying micrometastases were *KS1/4*-positive *LUNX*-positive, respectively (Wallace et al., 2005). Another study observed a 100% expression of *KS1/4* in the mediastinal lymph nodes of patients with NSCLC (Mitas et al., 2003a). However, in our study, only 25% of the NCSLC patients were *KS1/4*-positive. In addition, *KS1/4* mRNA was detected in the peripheral blood of 2/15 (13.3%) of patients with BPD. Therefore, the expression of *KS1/4* mRNA must be tested in the peripheral blood in the future to indicate the presence of micrometastases in NSCLC patients.

In conclusion, the detection of *LUNX* mRNA expression in the peripheral blood of NSCLC patients was both highly sensitive and specific, and could therefore be a good biomarker to indicate micrometastases. The test for the expression of *CK19* mRNA showed the highest sensitivity and low specificity. Moreover, the applicability of testing for the expression of *KS1/4* mRNA remains to be determined.

As of today (April 15, 2015), the 32 NSCLC patients included in this study were non-shedding, with 2 relapsing during a follow-up examination. Therefore, the presence of micrometastases in the peripheral blood does not indicate eventual metastasis. A positive signal for mRNA biomarker could indicate that the cancer may have metastasized. Therefore, the patients must be subjected to aggressive treatment regimens to inhibit postoperative recurrence and metastasis. Moreover, dynamic testing may help clinicians individualize the treatment plans for NSCLC patients.

Conflict of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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