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Combination of multiple gene markers to detect circulating tumor cells in the peripheral blood of patients with non-small cell lung cancer using real-time PCR

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ABSTRACT. Our study aims to determine the clinical significance of human telomerase reverse transcriptase (hTERT), S-phase kinase-associated protein 2 (Skp2) and thyroid transcription factor-1 (TTF-1) mRNA expressions in peripheral blood (PB) of patients with non-small cell lung cancer (NSCLC). Real-time polymerase chain reaction was used to investigate the gene expressions of hTERT, Skp2, TTF-1 as in the PB of 60 patients with NSCLC and 20 benign lung diseases. Statistical analyses were performed to examine the correlation between the expression of these mRNA markers and the clinical pathological features of NSCLC. We found that hTERT, Skp2, and TTF-1 were overexpressed in the PB of NSCLC patients, and demonstrated high specificity as well as sensitivity when used for NSCLC diagnosis. Significant correlation was observed

between disease stage and the three markers (P < 0.05). This study suggests that the genes hTERT, Skp2, and TTF-1 play important roles in tumor genesis and development, and can be used as diagnosis markers in NSCLC patients. The expression of three markers in combination can significantly improve the sensitivity and accuracy of diagnosis relative to single marker diagnosis, and provides a reliable method to detect CTCs in the PB. Additionally, these markers can also be used as diagnostic markers for clinical stages of NSCLC.

Key words: Human telomerase reverse transcriptase; S-phase kinase-associated protein 2; Circulating tumor cell; Thyroid transcription factor-1; Non-small cell lung cancer

INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide, and the primary risk factor for lung cancer is smoking, which accounts for more than 85% of all lung cancer-related deaths (Ettinger et al., 2010). The WHO has categorized lung cancer into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) based on cancer biology, therapy, and prognosis. NSCLC accounts for more than 85% of all lung cancer cases, and includes 2 major types: non-squamous carcinoma and squamous cell carcinoma (Ettinger et al., 2010). Like other tumors, the major cause of high mortality in NSCLC is tumor metastasis, a process which initiates circulating tumor cells (CTCs) (Hou et al., 2011).

CTCs, first detected in 1869, are cancer cells that break away from primary solid tumors and metastasize into peripheral blood (PB) (Allard et al., 2004). CTCs that have successfully evaded the immune system can develop into metastasis tumor lesions and form circulating tumor embolus (Kats-Ugurlu et al., 2009). Numerous studies in the past have shown that CTCs may be used as a marker to predict disease progression and survival in metastatic patients (Cristofanilli et al., 2004; Cohen et al., 2008; de Bono et al., 2008), and possibly even during early-stages of cancer (Rhim et al., 2012). Therefore, detection of CTCs has the potential to guide and assess therapy effectiveness/ necessity even in the absence of detectable metastases, as well as to offer insights into mechanisms of drug resistance. With the advancements in molecular technology and quantification methods such as real-time polymerase chain reaction (RT-PCR) techniques, rare CTCs in the PB can be detected (Yu et al., 2013). The main advantage of the RT-PCR approach lies in its sensitivity, which is considered to be higher than the reported sensitivity of immune-mediated detection methods such as immunocytochemistry (Zieglschmid et al., 2005). Various studies have reported that multiple biomarkers for CTCs can be detected in different cancers.

One such biomarker is telomerase reverse transcriptase (hTERT), which is a major limiting factor for telomerase activity, plays important roles in cellular immortalization, tumorigenesis, and the progression of cancer (Daniel et al., 2012). This cancer-related gene has been used for detection of CTCs in various cancers, including NSCLC (Wang et al., 2002). Another potential biomarker, the S-phase kinase-associated protein 2 (Skp2), can drive cells to progress from G1 to S-phase of the cell division cycle. Several studies have indicated a relationship between Skp2 expression and the aggressiveness of malignant tumors. (Gstaiger et al., 2001; Signoretti et al., 2002; Osoegawa et al., 2004). Thyroid transcription factor 1 (TTF-1), which is a 38-kDa nuclear protein member of the NKx2 family of homodomain transcription factors, is selectively expressed in

the lung, thyroid and diencephalons (Chang et al., 2004). It has also been used to identify primary lung cancer or tumor metastasis (Pelosi et al., 2001).

In this study, to investigate the clinical significance of combined markers in the diagnosis and staging of NSCLC, we evaluated the expression of three cancer- related mRNA markers, hTERT, Skp2 and TTF-1 by RT-PCR to detect CTCs levels in PB samples of NCSLC patients and healthy controls. Furthermore, we investigated the correlation between the expressions of these mRNA markers and the clinical pathological features of NSCLC to determine the relationship between CTCs status and cancer progression. We additionally determined the efficacy of each individual mRNA marker as well a combination of all three to compare the sensitivity and accuracy.

MATERIAL AND METHODS

Patients and controls

Sixty patients undergoing treatment at the Affiliated Hospital of Jiangsu University (Zhenjiang, China) and Nanjing Chest Hospital (Nanjing, China) were recruited between July 2011 and June 2012. Individuals diagnosed with primary NSCLC via pathologic histology without other concurrent tumors were selected as the experimental group. The study was approved by the local ethics committee, and all patients provided written informed consent for the analysis. The clinical pathological features of all patients are listed in Table 1. Additionally, twenty 35-75 (median 52) year-old patients who were diagnosed with benign pulmonary lesions were selected as the control group.

Table 1. The clinical pathologic features of 60 NSCLC patients.

Patients	Number
Total number	60
Age (years)	40-80 (median 57)
≥60	40
<60	20
Gender	
Male	38
Female	22
Disease stages	
I + II	33
III + IV	27
Pathological type	
Squamous carcinoma	29
Adenocarcinoma	31
Differentiation grade	
Low	28
Middle-high	32
Smoking condition	
≥600 cigarettes	35
<600 cigarettes	25

Processing of blood samples

PB samples were collected from each subject using Vacutainers (Becton Dickinson, Rutherford, NJ). The first 1-2 mL of PB was discarded to avoid epithelial cell contamination. EDTA-Na was added as an anti-coagulant. Mononuclear lymphocyte cells were enriched using density gradient centrifugation, which removed red blood cells and serum. To increase the likelihood of isolating CTCs for analysis, we enumerated CTCs in 7.5 mL using Cell Search to identify patients

with 1 CTC/mL or more. CTCs were then isolated from the remaining blood samples by IE-FACS (Immunomagnetic enrichment and fluorescence-activated cell sorting). Immunomagnetic enrichment of CTCs isolation and enumeration has been previously described (Magbanua et al., 2013).

Real-time PCR

Total RNA was extracted from lysed CTCs using TRIzol reagent (Invitrogen), and the quantity and integrity were assessed by gel electrophoresis, and visualized with an UV-Vis spectrophotometer (BIO-TEK, USA. First strand cDNA was synthesized from 4 µg of total RNA using a first strand cDNA synthesis kit (Promega, Madison, WI, USA). All RT-PCR assays were performed on the ABI Prism 7700 Sequence Detection Instrument (Applied Biosystems). Primers for β-actin, hTERT, Skp2, and TTF-1 are listed in Table 2. The cycling parameters of all genes are as follows: holding at 95°C for 3 min; 40 cycles of: 95°C for 45 s, annealing temperature for 45 s, 72°C for 45 s; followed by extension at 7 °C for 8 min. Gene expression was considered negligible when the Ct (threshold cycle) value was greater than 40.

Table 2. Primer sequences and annealing temperatures for RT-PCR.

Marker	Sense primer	Anti-sense primer	Annealing temperature (°C)
β-actin	5'-CACGAAACTACCTTCAACTCC-3'	5'-CATACTCCTGCTTGCTGATC-3'	60
hTERT	5'-CGGAAGAGTGTCTGGAGCAA-3'	5'-GGATGAAGCGGAGTCTGGA-3'	58
Skp2	5'-AGTCTCTATGGCAGACCCTAGACC-3'	5'-TTTCTGGAGATTCTTTCTGTAGCC-3'	57
TTF-1	5'-TACCAGGACACCATGAGGAA-3'	5'-CGCCGACAGGTACTTCTGTT-3'	62

hTERT: telomerase reverse transcriptase, Skp2: S-phase kinase-associated protein 2, TTF-1: thyroid transcription factor-1.

Statistical analysis

All data were analyzed using the SPSS 16.0 software (Chicago, IL, USA). Chi-square analysis and Fisher's exact test were used to analyze the correlations. $P < 0.05$ was considered statistically significant.

RESULTs

Sensitivity and specificity of real-time PCR detection of multiple markers

Sensitivity of the assay was represented as the percentage of positive detection in NSCLC patients, while specificity was the percentage of negative detection in the control group. The positive predictive value was indicated by the percentage of NSCLC patients in positive samples, while the negative predictive value was the percentage of control patients in negative samples. The accuracy of RT-PCR detection was determined by both sensitivity and specificity. The details of the RT-PCR results are listed in Table 3. Sensitivities of hTERT, Skp2, and TTF-1 were 72, 67, and 60% respectively, while their specificities were 100, 95, and 90% respectively. When the three markers were used in combination, sensitivity and specificity were 80 and 95% respectively, both of which were higher than those of any single marker. The accuracy of hTERT, Skp2, and TTF-1 was 79, 74, and 68% respectively, whereas the accuracy of three markers in combination was 84%.

Table 3. Sensitivity, specificity, and accuracy of mRNA markers.

Marker	Sensitivity % (n/n)	Specificity % (n/n)	Positive predictive value % (n/n)	Negative predictive value % (n/n)	Accuracy % (n/n)
hTERT	72 (43/60)	100 (20/20)	100 (43/43)	54 (20/37)	79 (63/80)
Skp2	67 (40/60)	95 (19/20)	98 (40/41)	48 (19/40)	74 (59/80)
TTF	60 (36/60)	90 (18/20)	95 (36/38)	41 (18/44)	68 (54/80)
Skp2 + hTERT + TTF	80 (48/60)	95 (19/20)	98 (48/49)	59 (19/32)	84 (67/80)

hTERT: telomerase reverse transcriptase, Skp2: S-phase kinase-associated protein 2, TTF-1: thyroid transcription factor-1

Correlation between gene expression levels of potential biomarkers with clinical pathological features of NSCLC patients

Correlations between expression levels of mRNA markers with the clinical pathological features are summarized in Table 4. Significant correlation was observed between diseases stages and the three markers ($P < 0.05$). Expression of TTF-1 was significantly correlated to the pathological type of NSCLC. No significant correlation was observed between the gene expression level and age, gender, smoking, or differentiation grade of NSCLC patients. Additionally, consistency analysis was used to determine the correlation between mRNA expression levels of hTERT, Skp2, and TTF-1 in NSCLC patients. There was no significant difference between any two gene markers ($P > 0.05$, data were not shown).

Table 4. Correlation of mRNA markers with clinical pathological features of NSCLC patients.

Characteristics	hTERT		P value	Skp2		P value	TTF-1		P value
	P	N		P	N		P	N	
Age (years)									
≥60	28	12	0.695	26	14	0.699	22	18	1.000
<60	13	7		14	6		11	9	
Gender									
Male	28	10	0.649	22	16	0.554	20	18	0.592
Female	15	7		11	11		10	12	
Smoking									
≥600	21	14	1.000	20	15	0.529	20	15	0.693
<600	15	10		10	5		13	12	
Pathological type									
Squamous carcinoma	17	12	0.965	15	14	0.611	15	14	0.315
Adenocarcinoma	18	13		14	17		20	11	
Differentiation grade									
Low	18	10	0.886	16	12	0.861	13	15	0.605
Middle-high	20	12		19	13		17	15	
Disease stages									
I+II	11	22	**<0.001**	13	20	**0.007**	15	18	**0.011**
III+IV	22	5		20	7		21	6	

hTERT: telomerase reverse transcriptase, Skp2: S-phase kinase-associated protein 2, TTF-1: thyroid transcription factor-1; P: positive, N: negative.

DISCUSSION

A number of tumor-associated or epithelial-specific markers such as CK, Her2, CEA, MUC1, EpCAM, EGFR, hTERT, survivin, c-met, and FN1, have been used for detection of CTCs in multiple tumors (Shen et al., 2009; Tewes et al., 2009). The positive detection rate of a single marker ranges from 30 to 60% in PB of cancer patients based on previous studies (Yu et al.,

2013)(Yu, 2013). In this study, we qualitatively detected expression levels of hTERT, Skp2, and TTF-1 singly or in combination via RT-PCR in PB samples of NSCLC patients. As shown in Table 3, positive detection rate of the three markers was 72, 67, and 60%. As a major limiting factor for telomerase activity, hTERT plays important roles in cellular immortalization, tumorigenesis, and cancer progression. Studies have demonstrated that higher expression of hTERT is present in tumor cells as compared with that in normal cells. Furthermore, hTERT has also been reported as an important marker for diagnosis and prognosis in different tumors including NSCLC (Wang et al., 2002; Shen et al., 2009; Yu et al., 2013). For TTF-1, the positive detection rate determined in this study is relatively higher than previous studies in NSCLC (Yoon et al., 2011; Yu et al., 2013). TTF-1 is a well-known marker that is used to distinguish adenocarcinoma from squamous cell carcinoma in lung cancer (Wu et al., 2003). We found that while squamous cell carcinomas showed increased levels of TTF-1 in PB, this may be due to tumor-induced damage of lung tissues, preexisting conditions, or the immune response. It has been proposed that expression of Skp2 may be related to the development and progression of NSCLC, and can be used as a prognostic marker in NSCLC patients (Takanami, 2005). However, we are the first to report specific positive detection rate of Skp2 expression in PB. Thus, hTERT, Skp2, and TTF-1 genes may play important roles in tumor genesis and development, and serve as diagnosis markers in NSCLC patients.

To the best of our knowledge, it is the first time that hTERT, Skp2, and TTF-1 have been used in combination to detect CTCs in NSCLC patients. The sensitivity and accuracy of the three markers in combination were 80 and 84%, respectively. The result is similar to the study by Yu et al. (2013) in lung adenocarcinoma and slightly higher than the result of Sher et al. (2005) in NSCLC patients . We found that when the three markers are analyzed in combination, the positive detection rate demonstrated a 10% increase compared with single marker analysis. These results suggest that detection of hTERT, Skp2, and TTF-1 in combination can significantly improve the sensitivity for NSCLC diagnosis compared with single markers.

To investigate the clinical application of each marker, we compared the expression levels of the three mRNA markers with the clinical pathological features in NSCLC patients. To ensure independent expression of the markers, correlation between mRNA expression of hTERT, Skp2 and TTF-1 was also calculated. We did not find any correlation between any two gene markers ($P > 0.05$) was observed. As shown in Table 4, expression of hTERT, Skp2, and TTF-1 all showed significant correlation with disease stages. The expression level of all three markers in III + IV stage was significantly higher than that in I + II stage ($P < 0.05$). In particular, hTERT represented higher sensitivity than the two other markers in the diagnosis of clinical stages of NSCLC ($P < 0.001$). This was similarly observed in previous lung cancer studies (Wang et al., 2002; Miura et al., 2006). In addition, expression level of the three mRNA markers did not show any correlations with age, gender, smoking, and differentiation grade of NSCLC patents. These results indicate that Skp2, TTF-1, and especially hTERT, can be used as diagnostic markers for clinical stages of NSCLC. It is possible that hTERT, Skp2 and TTF-1 are all differentially associated with disease prognosis, and may be used to predict the aggressiveness and metastasis of primary NSCLC.

In conclusion, we were able to detect hTERT, Skp2, and TTF-1 mRNA in PB of NSCLC patients, which may provide a reliable method of detecting CTCs in PB. Combination detection of multiple markers is correlated with TNM stages of NSCLC, and can be used as a diagnosis method for NSCLC and a predictor for tumor progression. However, further research needs to be conducted in order to assess the clinical value of these potential biomarkers and to determine the possible mechanisms by which they affect NSCLC.

Conflicts of interest

The authors declare no conflict of interest.

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