



Detection of SHOX2 DNA methylation by methylation-specific PCR in non-small cell lung cancer

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Background: Lung cancer is the leading cause of cancer-related death worldwide. Short stature homeobox 2 (SHOX2) methylation detected by real-time polymerase chain reaction (PCR) has recently been demonstrated to be a potential biomarker in the diagnosis of lung cancer. However, more cost-effective methods are still needed to help cancer detection in the early stage of lung cancer. The aim of this study was to examine the methylation status of the *SHOX2* gene and to investigate its diagnostic value in non-small cell lung cancer (NSCLC) patients.

Methods: A total of 89 Chinese NSCLC patients and 9 non-tumor patients was enrolled in this study. The methylation status of *SHOX2* gene in NSCLC tumor tissues/corresponding non-neoplastic lung tissues and lung tissues from non-tumor patients was examined by methylation-specific PCR (MSP).

Results: We found that SHOX2 methylation was significantly associated with NSCLC ($P=0.003$). We also analyzed the correlation of SHOX2 methylation with clinicopathological variables including sex, age, tumor pathologic classification, tumor differentiation degree, TNM stage, T stage, and nodal status, and found no significant correlation between them.

Conclusions: These results suggested that *SHOX2* gene methylation was closely associated with lung carcinogenesis. Thus, SHOX2 methylation could be used as a potential marker to help NSCLC detection. MSP might be used as a cost-effective method alternative to real-time PCR in detection of SHOX2 methylation in the early diagnosis of NSCLC.

Keywords: Stature homeobox 2 methylation (SHOX2 methylation); non-small cell lung cancer (NSCLC); diagnosis; methylation-specific PCR (MSP)

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (1). In China, lung cancer is the most prevalent cancer type and accounted for 25.2% and 29.5% of the total

cancer incidence and mortality in 2012, respectively (2). Over one million people die from lung cancer globally every year, among which approximately 0.6 million are from China. Worse yet, the incidence of lung cancer is still increasing in China due to the high prevalence of cigarette

use and other factors (3).

Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers and carries a dismal 5-year survival rate of 15% (3). Nevertheless, the International Early Lung Cancer Action Program Investigators reported that, among 302 participants with clinical stage I lung cancer who underwent surgical resection within 1 month after diagnosis, the survival rate was 92% (4). These results suggest that timely detection of lung cancer and appropriate treatment may prevent or interrupt cancer progression and improve overall survival rate. However, more than 75% of lung cancer patients are diagnosed at a locally advanced stage or with metastatic disease, partially due to the lack of an effective screening program for early detection (5). Therefore, simple, safe, and cost-effective methods to aid in early detection of lung cancer are greatly needed.

Tumor markers are molecules specifically expressed by tumor cells or produced by the host in response to tumors. Epigenetic changes of certain genes, such as DNA methylation, could occur at early stage of carcinogenesis, thus could be used as a type of tumor marker. DNA methylation based diagnostic devices has been used in screening of malignant diseases including colorectal cancer (6). *SHOX2* gene is located on human chromosome 3. *SHOX2* gene is widely expressed in tissue including skeleton, branchial arches, heart tissue and central nervous system (7). *SHOX2* methylation is closely related to tumor occurrence and progression. In colorectal cancer, *SHOX2* methylation could be used as a powerful biomarker for cancer screening and post-therapeutic monitoring (8). *SHOX2* gene methylation status could also be used as potent prognostic parameter for predicting patient survival in lower-grade glioma patients (9). Recently, Dietrich *et al.* reported that *SHOX2* methylation is a reliable biomarker for the diagnosis of lung cancer (10-14). Based on this potential, *SHOX2* methylation has been developed as a commercially available assay, the Epi proLung BL Reflex Assay, for early lung cancer detection (13).

Specific DNA methylation can be measured by several polymerase chain reaction (PCR)-based methods, including methylation-specific PCR (MSP) and real-time quantitative PCR. The aforementioned commercial *SHOX2* methylation assay detects *SHOX2* methylation through the generation of bisulfate-converted template DNA followed by real-time PCR using TaqMan probes (13). While this is sensitive and quantitative, it is also relatively expensive.

Compared with real-time PCR-based methods, MSP is much more cost-effective. It is also fast, sensitive, and specific, and has been widely used for DNA methylation analyses (15,16). Therefore, we chose to investigate the diagnostic value of *SHOX2* methylation detection by MSP in lung cancer as this method may reduce costs and thus may facilitate the use of *SHOX2* methylation in large-scale lung cancer screening, especially in developing countries where cost is a limiting factor. Specifically, we examined *SHOX2* methylation by the MSP method in tumor tissue and corresponding non-tumor lung tissue from a cohort of Chinese NSCLC patients, and further analyzed the diagnostic value of *SHOX2* methylation measured by MSP in NSCLC detection. We present the following article in accordance with the STARD reporting checklist (available at <http://dx.doi.org/10.21037/tcr-20-887>).

Methods

Patients

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The patients underwent surgery after providing informed consent, and the requirement for the individual consent of patients whose records were evaluated was waived because they were de-identified in the study. The study was approved by institutional ethics board of China-Japan Friendship Hospital (No. 2018-13-K08). A total of 89 NSCLC patients and 9 non-tumor patients were included in this study.

Clinicopathological characteristics of the participating patients with cancer or non-cancer are presented in *Tables 1* and *2*, respectively. All tumors in these patients were completely resected (R0 category) by surgery. Additionally, histologically normal lung tissue specimens obtained at surgery from 9 patients with no evidence of cancer were used as a control group (including 8 patients with pulmonary bulla and 1 patient with benign tumor). In the control group, 6 patients were men and 6 were under 60 years old.

Tissue acquisition and DNA extraction

Tissues for DNA analysis were obtained immediately after lung resection before starting mediastinal lymphadenectomy and were frozen in liquid nitrogen. Tissues were analyzed as tumors or uninvolved lung tissues taken from the farthest

Table 1 Clinicopathological characteristics of 89 non-small cell lung cancer patients

Characteristics	N (%)
Sex	
Male	63 (70.8)
Female	26 (29.2)
Age*	
<60	42 (47.2)
≥60	47 (52.8)
Pathologic classification	
Squamous cell carcinoma	34 (38.2)
Adenocarcinoma	47 (52.8)
Other	8 (9.0)
Differentiation degree	
Undifferentiated/poorly	39 (43.8)
Moderately/well	50 (56.2)
TNM stage	
I/II	52 (58.4)
III/IV	37 (41.6)
T stage	
T1/2	58 (65.2)
T3/4	31 (34.8)
Nodal status	
N0	39 (43.8)
N+	50 (56.2)

*, age according to median value of 60 years (range 36–78). T, tumor size; N, total number of patients.

Table 2 Clinicopathological characteristics of 9 non-cancer patients

Characteristics	N
Sex	
Male	6
Female	3
Age	
<60	6
≥60	3

distance from tumors. Ten μm thick frozen sections were taken from blocks of tumor tissues and stained with H&E for histopathological evaluation. Sections were pooled for analysis from areas of estimated 75% malignant cells. Genomic DNA was isolated from frozen tissue by standard methods of proteinase K digestion and phenol-chloroform extraction using Tiangen Cells and Tissue DNA Isolation Kit (Tiangen Biotech Co., LTD, Beijing, China) according to the manufacturer's instructions.

Detection of SHOX2 methylation using MSP

A total of 500 ng genomic DNA extracted from tissue samples was modified by sodium bisulfite with DNA Methylation Detection Kit (BioChain, USA) according to the manufacturer's instructions. 7 μL modified DNA was used for PCR in a total reaction volume of 25 μL . PCR program was set as 35 cycles of melting (95 °C for 30 s), annealing (58 °C for 30 s) and extension (72 °C for 30 s). The 25 μL reaction mixture contained 1 \times PCR Buffer (Mg^{2+} Plus), 200 μM of each dNTP, 0.5 μM forward primer, 0.5 μM reverse primer, and 0.5 unit of GoTaq Hot Start Polymerase (Promega, USA). PCR products were separated in 3% agarose gel supplemented with ethidium bromide. DNA was visualized under UV illumination. SHOX2 was recorded as methylated if MSP amplification products were detected in both reactions with unmethylation and methylation primers, or with methylation primers only. The primer sequences are as follows:

- ❖ Unmethylation-specific primers: TGTATGAGTATAGGTGTTTATG (forward), AAAACAATTACTTTCACCCA (reverse);
- ❖ Methylation-specific primers: CGTACGAGTATAGGCGTTTACG (forward), AAAACGATTACTTTCGCCCCG (reverse).

Statistical analysis

Statistical comparisons were performed using either the χ^2 -test or Fisher's exact test, as appropriate. Student's *t*-test was performed to assess the statistical significance between the mean values. All data were analyzed by SPSS software (SPSS Statistics 19, Rel. 19.0. 2010. Armonk, NY: IBM Corp.). P values less than 0.05 were considered to be statistically significant.

Results

The characteristics of the NSCLC patients

A total of 89 Chinese NSCLC patients were included in this study, consisting of 63 (70.8%) men and 26 (29.2%) women. Forty-two (47.2%) patients were younger than and 47 (52.8%) patients were older than the age of 60, which was the median value (range 36–78) of the cohort of patients. Based on the pathologic classification, 34 (38.2%) patients had squamous cell carcinomas, 47 (52.8%) had adenocarcinomas, and 8 (9.0%) had tumors of other histological types. The primary tumors were graded histopathologically into undifferentiated/poorly-differentiated tumors and moderately/well-differentiated tumors, with 39 (43.8%) patients classified into the former group and 50 (56.2%) into the latter one. Fifty-two (58.4%) patients had stage I/II tumors and 37 (41.6%) had stage III/IV tumors. Fifty-eight (65.2%) tumors were graded at T1/T2 stage and 31 (34.8%) were at T3/4 stage. Tumors in 39 (43.8%) patients had not metastasized while 50 (56.2%)

patients had nodal metastasis.

Correlation of SHOX2 methylation with NSCLC tumor tissues

We determined the methylation status of SHOX2 in tumor tissues and paired non-neoplastic lung tissues of 89 Chinese NSCLC patients and in the lung tissues of 9 non-cancer patients using MSP (Table 3). Representative DNA gel electrophoresis image of polymerase chain reaction products was showed on the Figure 1. SHOX2 methylation was detected in tumor tissue samples from 83 of the 89 NSCLC patients, with an occurrence of 93.3%. Conversely, SHOX2 methylation was found in only 6 (6.7%) of the paired non-neoplastic lung tissue samples. Fisher’s test showed that there is a significant correlation between SHOX2 methylation and tumor tissue, with a P value of 0.003. We did not detect SHOX2 methylation in the lung tissues of any of the 9 non-tumor patients. These results suggest a promising diagnostic value of detection SHOX2 methylation by MSP in NSCLC patients.

Table 3 SHOX2 DNA methylation in non-small cell lung cancer and corresponding non-neoplastic lung tissues as well as in lung tissues of non-cancer patients

Variable	Methylation status		P*
	Tumor	Non-neoplastic lung tissue	
NSCLCs: SHOX2 M/U (n=89)	83/6	6/83	0.003
Non-cancer patients: SHOX2 M/U (n=9)		0/9	

*, statistical analysis performed by Fisher’s or χ^2 test, comparing the number of patients with methylated gene (M) with the number of unmethylated gene (U) detected. M, number of patients with methylation detected. U, number of patients without methylation detected. NSCLC, non-small cell lung cancer.

Association of SHOX2 methylation with clinicopathological variables in Patients with NSCLCs

We also analyzed the association between SHOX2 methylation and the clinicopathological variables in NSCLC patients using Fisher’s or χ^2 test (Table 4). Results showed that there are no obvious statistically significant correlations between SHOX2 methylation and any of the collected clinicopathological variables including sex, age, tumor pathologic classification, tumor differentiation degree, TNM stage, T stage and nodal status. These results suggest that SHOX2 methylation is significantly associated with NSCLC tumor tissue regardless of clinicopathological status. These results further support the diagnostic value of SHOX2 methylation in NSCLC patients.

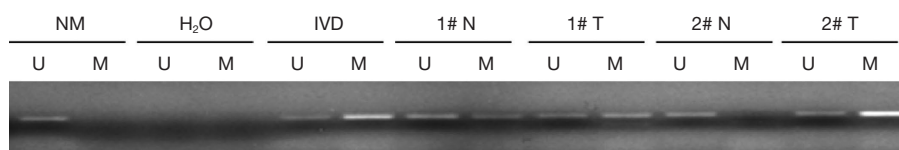


Figure 1 Representative DNA gel electrophoresis image of polymerase chain reaction products. NM, lymphocyte DNA as unmethylation positive control; IVD, *in vitro* methylated lymphocyte DNA as methylation positive control; T, tumor tissue; N, non-neoplastic lung tissue; U, MSP with unmethylation primers; M, MSP with methylation primers; 1#, 2# represent different non-small cell lung cancer patients.

Table 4 Association of SHOX2 DNA methylation detected in non-small cell lung cancer patients with clinicopathological variables using Fisher's or χ^2 test

Characteristics	SHOX2 M/U	
	Tumor	Non-neoplastic lung tissue
Sex		
Male	58/5	4/59
Female	25/1	2/24
P ^a	0.67	1.00
Age*		
<60	41/1	2/40
≥60	42/5	4/43
P ^a	0.21	0.68
Pathologic classification		
Squamous cell carcinoma	32/2	2/32
Adenocarcinoma	43/4	3/44
Other	8/0	1/7
P ^a	0.65	0.79
Differentiation degree		
Undifferentiated/poorly	38/1	1/38
Moderately/well	45/5	5/45
P ^a	0.23	0.23
TNM stage		
I/II	48/4	5/47
III/IV	35/2	1/36
P ^a	1.00	0.39
T stage		
T1/2	55/3	4/54
T3/4	28/3	2/29
P ^a	0.42	1.00
Nodal status		
N0	36/3	3/36
N+	47/3	3/47
P ^a	1.00	1.00

P^a statistical analysis performed by Fisher's or χ^2 test, comparing the number of patients with methylated SHOX2 (M) with the number of unmethylated SHOX2 (U) detected. *, age according to median value of 60 years (range 36–78). M, number of patients with methylation detected. U, number of patients without methylation detected.

Discussion

In this study, we applied MSP to detect the methylation status of the *SHOX2* gene in tumor tissues and corresponding non-tumor lung tissues in 89 Chinese NSCLC patients and found that *SHOX2* methylation is significantly correlated with NSCLC. This findings are consistent with prior studies (10,11) and support that *SHOX2* methylation may be a useful biomarker for lung cancer diagnosis.

At present, both qualitative and quantitative methods are used to measure DNA methylation of specific genes. Despite the lack of quantitative information obtained from real-time PCR based methods, MSP is widely applied in clinical researches for DNA methylation because it is fast, simple, specific, requires resources commonly available in a molecular genetics laboratory, and is very cost-effective (16-22). MSP is also sensitive in that it can detect as low as 0.1% (50 pg of methylated DNA out of 50 ng of total DNA) to 1% (0.1 ng of methylated DNA out of 10 ng of total DNA) methylated alleles (23-25). By using Quantitative HeavyMethyl (HM) real-time PCR, Dietrich *et al.* reported hypermethylation of the *SHOX2* locus in tumor tissue in 96% of tumors from a group of 55 patients with lung cancer (12), where as in our current study, *SHOX2* methylation was detected in 93.3% tumor tissues from the cohort of 89 NSCLC patients. These results suggest that MSP may be used as a cost-effective alternative in detection of *SHOX2* methylation in the diagnosis of NSCLC. The development of this simple, low-cost MSP method may facilitate the wide use of detection of *SHOX2* methylation in large-scale NSCLC early screening, especially in developing countries.

DNA methylation is an important epigenetic modification in cancer. Aberrant methylation of the promoter region in a specific gene causes gene silencing. Hence, methylation of tumor suppressor genes is an early and frequent event during carcinogenesis (26). *SHOX2* is a member of the homeobox family and plays important roles in embryonic morphogenesis, heart, skeleton, and nervous system development (27-35). In addition to its involvement in early development, human *SHOX2* has been found to be expressed in tumors including neuroblastomas and breast cancer (36,37). Although *SHOX2* methylation is demonstrated to be an excellent biomarker in lung cancer diagnosis, whether the *SHOX2* gene qualifies as a tumor suppressor gene is not clear. Interestingly, unlike hypermethylated tumor suppressor genes that are down-regulated in tumors, elevated *SHOX2* expression

was reported to be associated with tumor recurrence of hepatocellular carcinoma (38). However, the mechanism of increased expression of SHOX2 in hepatocellular carcinoma is unknown. Dietrich *et al.* found that SHOX2 was expressed at similar transcriptional levels in tumor tissue and normal adjacent tissue from lung cancer patients (12). The authors further reported that SHOX2 methylation was correlated with SHOX2 gene amplification (12) caused by genomic gain of chromosome 3q involving the SHOX2 gene, which has been recognized as one of the most prevalent and significant chromosomal rearrangements in lung cancer (39,40). Therefore, the up-regulation caused by SHOX2 gene amplification may counteract the negative regulatory effect associated with SHOX2 methylation, and thus no significant expression change was detected between lung tumor and paired non-tumor lung tissues. Furthermore, the consistency of SHOX2 methylation observed in lung cancer tissue from Chinese patient samples reported herein and from Dietrich's work in European people (12) suggests that the epigenetic modification of SHOX2 seems similar in populations with different genetic backgrounds. The conflicting results of the expression of SHOX2 in hepatocellular carcinoma and lung cancer indicate that a lot of work remains to be done to clarify the relationship between SHOX2 expression and methylation in different tumors, as well as the molecular mechanisms of SHOX2 regulation.

Appropriate usage of SHOX2 methylation to aid in the early detection and diagnosis of lung cancer will facilitate timely treatment of lung cancer patients to improve their survival. Our results demonstrated that the MSP method provides comparable results in detection of SHOX2 methylation when compared with the much more expensive real-time PCR based method.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics board of China-Japan Friendship Hospital (No. 2018-13-K08). The patients underwent surgery after providing informed consent, and the requirement for the individual consent of patients whose records were evaluated was waived because they were de-identified in the study.

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