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Original Article

ROS1 gene rearrangement and clinicopathological characteristics in Chinese NSCLC patients

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Abstract: Objective: Since Rikova et al. reported c-ros oncogene 1 (ROS1) rearrangements in non-small cell lung cancer (NSCLC) in 2007, data on the clinicopathological characteristics of ROS1-positive patients in China are scarce. We aim to examine the correlation between clinicopathological characteristics of NSCLC patients and the frequency of ROS1-rearrangements. Methods: The cancer tissues of 1720 patients with NSCLC were analyzed using fluorescence in situ hybridization (FISH) assay to assess the presence of ROS1 gene fusions. Polymerase Chain Reaction (PCR) direct sequencing was performed to identify the fusion genes in positive tissues. Clinicopathological characteristics of the patients and the corresponding frequency of ROS1-rearrangement were analyzed. Results: Among the 1720 NSCLC patients, 31 (1.8%) were tested positive for ROS1-rearrangement. Compared to the ROS1-negative group, they were significantly younger and more likely to be never-smokers (each $P<0.05$). All of the ROS1-positive tumors were adenocarcinomas, and tend to be higher grade cancer ($P<0.05$), however there was no significant preference in gender ($P>0.05$). Four ROS1 fusions were observed in the samples, they were CD74-ROSl (n=9), SLC34A2-ROSl (n=7), SDC4-ROSl (n=8) and TPM3-ROSl (n=7). Conclusions: ROSl-rearrangements were recognized in 1.8% of the Chinese NSCLC patients studied, similar to the prevalence of 1-2% that had been-reported. The clinicopathological characteristics of these patients were clearly associated with ROS1-rearrangements. Specifically, ROS1-rearrangements were significantly more prevalent in the younger and never-smoking lung adenocarcinoma patients.

Keywords: Non-small cell lung cancer, tyrosine kinase inhibitor (TKI), fluorescence in situ hybridization (FISH), ROS1 fusion

Introduction

Lung cancer is known to be the leading cause of cancer-related mortality worldwide [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers [2]. Approximately one third of NSCLC patients suffer from the locally advanced stage of the disease at the time of diagnosis [3]. Although the prognosis of advanced NSCLC is very poor, recent development of targeted therapy has emerged as a highly effective treatment for these patients. The best-known target drug is epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), such as erlotinib, which are particularly effective for treating patients with EGFR mutation [4, 5]. More recently, an excellent and effective anaplastic lymphoma kinase (ALK) inhibitor, crizotinib, has been used for the treatment of NSCLC patients with ALK gene rearrangement. ALK-rearrangement is found in approximately 3-6% of NSCLC patients and the overall response rate rises to 57% in ALK-rearranged patients treated with crizotinib [6, 7].

ROS1 encodes a transmembrane tyrosine kinase receptor, which belongs to the insulin receptor family, which has high homology in its protein kinase domain with ALK [8]. The ROS1-rearrangement was first discovered in NSCLC in 2007 and ~2% of NSCLC patients carried ROS1 fusion [9]. In lung cancer, several fusion partners of ROS1 have been identified, include FIG,

CD74, SLC34A2 and SDC4, which lead to oncogenic transformation and constitutive kinase activity in cell culture and/or *in vivo* [10, 11]. Due to highly similar tyrosine kinase domains, experiments *in vitro* indicate that crizotinib can inhibit the growth and induce apoptosis of HCC78, which is the NSCLC cell line that demonstrates the SLC34A2-ROS1 fusion without ALK-rearrangement [12]. In recent clinical studies, advanced NSCLC patients with ROS1-rearrangements have derived great benefit from crizotinib treatment [13].

In this study, we determine the prevalence of ROS1-rearrangements in NSCLC via fluorescent in situ hybridization (FISH), identify the variants of ROS1 fusion genes by direct sequencing, and define the clinicopathological characteristics of ROS1-positive NSCLC patients. Our data indicate that ROS1-rearrangements arise significantly in younger and never-smoking lung adenocarcinoma patients in China.

Materials and methods

Study population and tissue microarray (TMA) construction

All included 1720 cases received curative surgery at the Union Hospital, Huazhong University of Science and Technology (HUST), Wuhan, China, from January 2009 to January 2014. Their medical records were reviewed to extract data of clinicopathological characteristics, including age, sex, cancer stage, histology, and smoking history. For pathological diagnosis and staging of tumors, we referred to the 2004 World Health Organization (WHO) classification, the tumor-node-metastasis staging system of the International Association for the Study of Lung Cancer (version 7), and the 2011 IASLC/ATS/ERS proposal.

Tumor tissues of the 1720 patients were collected, formalin-fixed and paraffin-embedded. After hematoxylin and eosin staining, TMA blocks were built using Quick-ray manual tissue microarrayer (Unitma Co., Ltd., Seoul, Korea) to perform FISH assays. This study has been approved by the institutional Research Medical Ethics Committee of Union Hospital, and all participants have provided written informed consent for the genetic analysis.

Fluorescent In situ hybridization

A break-apart FISH probe (ZytoLight SPEC ROS1 Dual Color Break Apart Probe, ZytoVision GmbH, Bremerhaven, Germany) was used according to the manufacturer's instructions. FISH measurements were performed using an Olympus BX43 TRF microscope (Olympus, Tokyo, Japan) equipped with three filters (DAPI/Green/Red). The diagnostic criteria for ROS1-rearrangement were as follows: ① a minimum of 50 cells were evaluated; ② split signals or isolated green signals were detected; ③ rearrangement-positive cells constituted no less than 15% of the enumerated tumor cells.

RT-PCR and direct sequencing

RNA was purified from the ROS1-positive tissues using the RNeasy FFPE Kit (Qiagen, Hilden, Germany) according to the manufacture's protocol. PCR amplifications were performed with an Applied Biosystems 7500 Fast Dx Real-Time PCR instrument (life technologies, USA), using the OneStepPrimeScriptRT-PCR Kit (Takara, Japan) according to the manufacture's protocol. PCR reactions were performed to amplify either SLC34A2-ROS1, CD74-ROS1, TPM3-ROS1, SDC4-ROS1 or other according to the previously published primers [13, 14]. PCR reactions were performed under the following conditions: 42°C for 5 min, 95°C for 10 sec, 40 cycles with 95°C for 5 sec, 60°C for 30 sec, followed by a Sanger sequencing test of the PCR products.

Statistical analysis

Different statistical significance tests were used to examine the association between ROS1 gene fusion status and the clinicopathological features, like sex, age, and tumor stage. Fisher's exact test was used for sex, while linear-by-linear association tests for age and tumor stage. Independent samples tests were applied where appropriate in order to determine the association. All tests were two-sided, with a statistical significance $P<0.05$. The statistics analyses were performed with the SPSS statistics 17.0 software (SPSS Inc., Chicago, IL).

Results

Characteristics of patients with ROS1-positive NSCLCs

We successfully enrolled 1720 NSCLS cases and made the tumor tissues into a TMA panel. We screened all the samples in the TMA using a break-apart FISH assay. Details of the clinico-

Table 1. Demographics and Clinicopathological Characteristics of Patients With ROS1-Positive NSCLC

Clinical Characteristic	All patients (n=1720)		ROS1 positive (n=31)		ROS1 negative (n=1681)		*P* Value (ROS1 positive V ROS1 negative)
	No.	%	*No.*	%	*No.*	%	
Age, in years							
Median	62.3		48.5		63.1		P<0.001
Range	29-86		29~76		32~86		
Sex							
Male	895	52.03	19	1.10	876	50.93	P=0.30
Female	825	47.97	12	0.70	813	47.24	
Smoking history							
Never	782	45.47	27	1.57	755	43.82	P<0.001
Light-smoker	231	13.43	3	0.17	228	13.23	
Smoker	707	41.10	1	0.06	706	40.93	
Pathology							
Adenocarcinoma	964	55.95	31	1.80	933	54.02	P<0.001
Squamous	688	39.93	0	0	688	39.81	
Adenosquamous carcinoma	36	2.09	0	0	36	2.08	
Large cell carcinoma	32	1.85	0	0	32	1.85	
Stage							
I	428	24.75	2	0.12	426	24.60	
II	380	21.98	4	0.23	376	21.70	
III	468	27.07	11	0.63	457	26.36	P=0.001
IV	444	25.64	14	0.81	430	24.78	

Abbreviations: NA, not available; NS, not significant; NSCLC, non-small-cell lung cancer.

pathological characteristics of these patients are listed in **Table 1**. We observed 31 ROS1 FISH-positive NSCLCs in the 1720 cases, with a prevalence rate of 1.8%. All the 31 NSCLCs were pulmonary adenocarcinoma. The sex ratio in the ROS1 FISH-positive group was male:female =19:12, compared to 272:271 in the ROS1 FISH-negative group. Although gender preference appeared to be more obvious in the positive-patient group than in the negative one, this finding does not reveal any statistically significant difference (P=0.30). The median age of the ROS1 FISH-positive patients tend to be lower than the ROS1 FISH-negative ones (57 vs 61 years), though it has no significant difference (p=0.867). Most of the positive patients presented with stage III (n=11) or stage IV (n=14) disease at diagnosis. 27 of the positive patients (87.1%) were never-smokers, whereas 3 were active smokers, with 1 patient having a 20 years smoking history.

ROS1-rearrangement patterns

In the FISH analysis, both split signals and single green signals were observed. The number of cells showing abnormal signals ranged from 31% to 95% of the total number of cells in a sample. In most of the positive cases aberrant signals were observed, as previously reported [14]. However, for some of the ROS1-positive tumor cells, we observed a certain variation in the signal patterns. Some of the tumor cells showed only additional green signals and no (or fewer) split signals, indicating tumor cell heterogeneity. Some cells showed multi-split signals (see **Figure 1**).

Direct sequencing analysis of ROS1 rearrangement

To identify ROS1 fusion partner genes, we examined the positive samples using RT-PCR followed by direct sequencing. We found that seven out of the 31 positive samples had SLC34A2-ROS1 fusion, nine had CD74-ROS1, eight had SDC4-ROSl and seven had TPM3-ROS1 fusion. We observed two kinds of SLC34A2-ROS1 fusion, namely SLC34A2 exon 4 fused to ROS1 exons 32 and 34, respectively. The other gene fusions observed in this study showed only short transcript. They were CD74

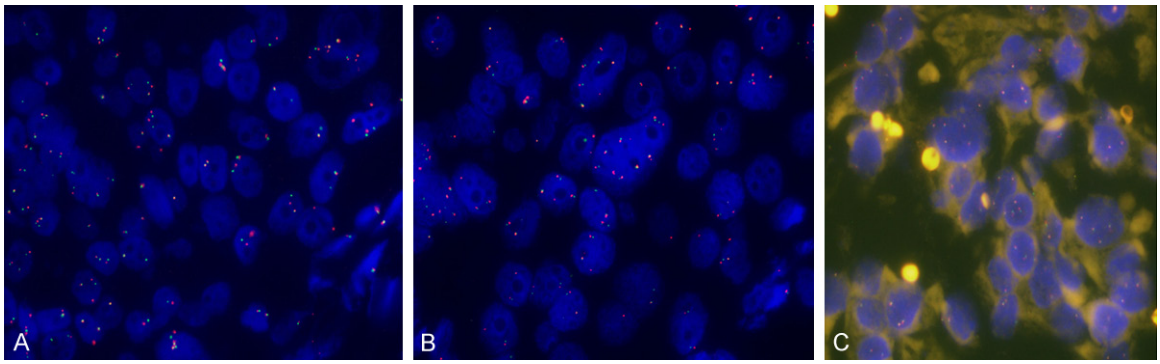

Figure 1. FISH for detection of ROS1-rearrangement in the NSCLC tissues. A. FISH-positive cells showing a split signal or an isolated green signal per cell. B. FISH-positive cells showing a split signal or a multi-split signal per cell. C. FISH-negative cells showing an intact two-fused signal per cell.

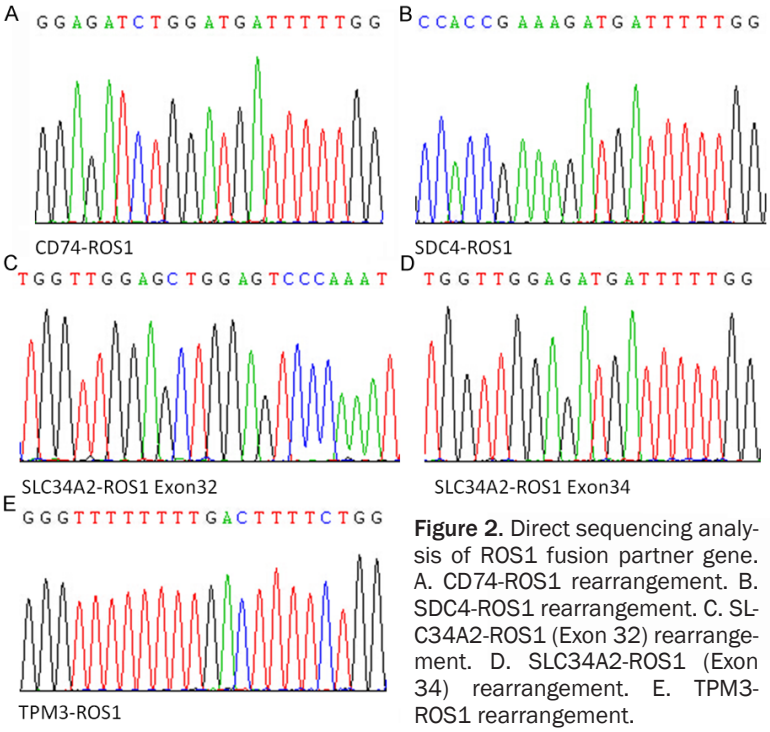

Figure 2. Direct sequencing analysis of ROS1 fusion partner gene. A. CD74-ROS1 rearrangement. B. SDC4-ROS1 rearrangement. C. SLC34A2-ROS1 (Exon 32) rearrangement. D. SLC34A2-ROS1 (Exon 34) rearrangement. E. TPM3-ROS1 rearrangement.

exon 6 and SDC4 exon 2 fused to ROS1 exon 34, and TPM3 exon 8 fused to ROS1 exon 35 (see **Figure 2**).

Discussion

Chromosomal rearrangements of ROS1 receptor tyrosine kinase gene have emerged as an important predictive biomarker for the use of ALK-targeted inhibitor 'crizotinib' for NSCLC [15, 16]. The clinicopathological characteristics of ROS1-rearrangement patients have thus become a topic of interest. The aim of this study was to determine the relation between ROS1-rearrangements and the clinicopathological characteristics of NSCLC, and to provide guidance for future clinical treatment of NSCLC patients.

The oncogenic ROS1 fusion is present in 1-2% of NSCLC cases [17, 18], and is likely to be specific for adenocarcinoma [19]. In this study we have measured 1720 NSCLC tissues by FISH assay, and identified approximately 1.8% (31/1720) of them harboring ROS1-rearrangement. With an estimated 520,000 new cases of NSCLC per year in China, we anticipate that there are 9,880 new ROS1-fusion positive patients per year, who would benefit from targeted inhibitors (such as 'crizotinib') treatment. All the 31 patients studied were confirmed ROS1-fusion positive by RT-PCR and direct sequencing, which suggested FISH is an effective diagnostic technique for detecting ROS1 chromosomal rearrangements in tumor tissues. Because RT-PCR and direct sequencing theoretically failed to detect all the previously described or some undiscovered rearrangements in a substantial number of FISH-positive cases, and thus may not be the optimal biomarker assay. On the contrary, FISH is an effective method for detecting ROS1-rearrangement, albeit FISH analysis is often cumbersome and expensive, requiring sophisticated equipment,

skilled personnel, well-preserved FFPE samples, sufficient cancer cells, etc.

Although there might be a selection bias considering that an excessive number of the biopsied cases were stage III and IV, the ROS1-fusion positive group showed more frequent occurrence of advanced tumor stage than did the ROS1-fusion negative group. Similar to that of ALK-rearranged NSCLCs [13], ROS1-fusion positive NSCLCs occurred mostly in certain subgroups: patients of young age, of nonsmoking history, and with adenocarcinoma. The similar correlation between rearrangement occurrence and characteristics suggests that the two genetic subtypes may share a common pathogenesis, possibly environmental or genetic risk factors.

Due to the same correlation between pathological features and high degree of homology for ALK and ROS1, ROS1-rearrangements, similar to ALK-rearrangements, may fuse the kinase-domain containing 3' regions of tyrosine kinases to the 5' regions of unrelated genes [13]. The ROS1 gene fuses to at least nine partner genes, including those first found in glialblastoma. They are FIG-ROS1 and CD74-ROS1, SLC34A2-ROS1, TPM3-ROS1, SDC4-ROS1, EZR-ROS1, LRIG3-ROS1, KDELR2-ROS1 and CCDC6-ROS1 [13, 14]. Using an inverse PCR and direct sequencing technique, we identified nine cases with CD74-ROS1, seven with SLC34A2-ROS1, eight with SDC4-ROS1 and seven with TPM3-ROS1 fusion in the 31 ROS1-fusion positive NSCLC cases. Typically there are either one or two fusion patterns for a ROS1 fusion. We observed two kinds of SLC34A2-ROS1 fusion, which are SLC34A2 exon 4 fused to ROS1 exons 32 and 34, respectively. No ROS1 long transcript was identified in our cohort. However, Fu et al. reported three SDC4-ROS1 fusion patterns with long and short transcript including SDC4 Exon2 fused to ROS1 Exon32, SDC4 Exon4 fused to ROS1 Exon32 and SDC4 Exon4 fused to ROS1 Exon34 [19].

In summary, we have found that approximately 1.8% of the NSCLC patients examined harbor ROS1-rearrangements, which are more prevalent with younger and never-smoking lung adenocarcinoma patients.

Disclosure of conflict of interest

None.

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