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ORIGINAL RESEARCH

High feasibility of cytological specimens for detection of *ROS1* fusion by reverse transcriptase PCR in Chinese patients with advanced non-small-cell lung cancer

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Purpose: Our previous study demonstrated that cytological specimens can be used as alternative samples for detecting anaplastic lymphoma kinase (*ALK*) fusion with the method of reverse transcriptase PCR (RT-PCR) in patients with advanced non-small-cell lung cancer (NSCLC). The current study aimed to investigate the feasibility of cytological specimens for ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*) fusion detection by RT-PCR in advanced NSCLC patients.

Patients and methods: A total of 2,538 patients with advanced NSCLC, including 2,101 patients with cytological specimens and 437 patients with tumor tissues, were included in this study. All patients were screened for *ROS1* fusion status by RT-PCR. The efficacy of crizotinib treatment was evaluated in *ROS1* fusion-positive NSCLC patients.

Results: Among 2,101 patients with cytological specimens, the average concentration of RNA acquired from cytological specimens was 47.68 ng/μL (95% CI, 43.24–52.62), which was lower than the average of 66.54 ng/μL (95% CI, 57.18–76.60, P=0.001) obtained from 437 tumor tissues. Fifty-five patients harbored *ROS1* fusion gene that was detected by RT-PCR, and 14 of them were treated with crizotinib. The incidence of *ROS1* fusion was 1.95% (41/2,101) in 2,101 patients with cytological specimens, similar to the rate of 3.20% (14/437, P=0.102) for the 437 patients with tumor tissue. Regarding crizotinib treatment, no statistically significant differences were observed in the objective response rate (ORR) (81.8% vs 100%, P=0.604) between the cytological and tissue subgroups of *ROS1*-positive patients.

Conclusion: This study shows that cytological specimens can be utilized as alternative samples for *ROS1* fusion detection by RT-PCR in advanced NSCLC patients.

Keywords: non-small-cell lung cancer, ROS proto-oncogene 1 receptor tyrosine kinase, *ROS1*, cytological specimens, reverse transcriptase polymerase chain reaction, RT-PCR, crizotinib

Introduction

The identification of oncogenic mutations, such as in epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), and ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*), has raised great interest in small molecular tyrosine kinase inhibitors as therapeutics for non-small-cell lung cancer (NSCLC), with dramatic responses observed in patients harboring relevant driver mutations.[1–6] In particular, crizotinib, the first small molecular inhibitor targeting ROS1/ALK/MET to be tested in the clinic, has dramatically changed the therapeutic landscape for *ROS1* fusion-positive NSCLC.[5–8] Therefore, the detection of *ROS1* fusion status is a critical

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step in determining treatment strategy for this subgroup of patients.

ROS1 fusion gene represents a novel molecular subtype of NSCLC, accounting for ~1%–2.2% of NSCLC cases.[7–12] Several methods were performed to detect this fusion gene, including fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), and reverse transcriptase PCR (RT-PCR). FISH is considered to be the gold standard method for *ROS1* fusion detection in clinical trials. IHC is a cost-effective screening tool to identify *ROS1* fusion-positive NSCLC. In regards to *ROS1* molecular testing guideline, the recommendation of the College of American Pathologists/International Association for the Study of Lung Cancer/Association for Molecular Pathology guideline was that *ROS1* testing should be performed on all patients with advanced lung adenocarcinoma, irrespective of their clinical characteristics, and the Expert Consensus Opinion was that IHC may be used as a screening test for *ROS1* fusion status in patients with advanced lung adenocarcinoma. However, positive ROS1 IHC results should be reconfirmed by a molecular or cytogenetic method.[13] Both FISH and IHC are not limited to histological tissue, but also work with cytological specimens.[14–17] However, both methods require adequate quality and quantity of tumor cells; therefore, histological tissue is more suitable for screening than cytological specimens. However, advanced NSCLC patients are unsuitable for surgery or biopsy; in contrast, cytological specimens can be easily acquired. Several studies have shown that cytological specimens can be used for molecular testing in lung cancer.[18,19] In addition, our previous study detected *ALK* fusion status from cytological specimens in as many as 79% of the NSCLC patients.[20]

RT-PCR is another alternative screening method that is easy to perform and highly sensitive to detect *ROS1* fusion status. The Chinese Food and Drug Administration has approved the ADx *ROS1* fusion gene diagnostic kit (Amoy Diagnostics, Xiamen, China) for assessing *ROS1* fusion status in the clinic. Our previous studies reported a slightly higher incidence for *ROS1* fusion when detected by RT-PCR than through the FISH or IHC analysis methods used by other studies.[7–11,21] Furthermore, we have shown high feasibility for the detection of *ALK* fusion status from cytological samples by RT-PCR.[20] However, the feasibility of detecting *ROS1* fusion status from cytological specimens by RT-PCR remains unknown.

Hence, the purpose of this study was to investigate the feasibility of cytological samples as alternative specimens for *ROS1* fusion testing by RT-PCR in advanced NSCLC patients. We compared RNA yields and the incidence of *ROS1* fusion between cytological specimens and tumor tissue in 2,538 advanced NSCLC patients. Furthermore, we compared the efficacy of crizotinib treatment in *ROS1*-positive patients in light of different sample types.

Patients and methods

Patients and samples

This study included NSCLC patients who had histologically confirmed stage IV disease and were screened for *ROS1* fusion status by RT-PCR between October 1, 2013 and June 30, 2016 at Shanghai Pulmonary Hospital, Tongji University School of Medicine. Clinical data for each patient were collected in detail as described in our previous study.[8,22] Tumor responses were evaluated at 1 month after the first administration of crizotinib (250 mg, twice daily) and then after every two cycles thereafter on the basis of the Response Evaluation Criteria in Solid Tumors (version 1.1). An informed consent form was signed by each patient before the initiation of any study-related procedure. This study was approved by the Shanghai Pulmonary Hospital Ethics Committee. This study was conducted in accordance with the Declaration of Helsinki.

Specimen preparation and RNA extraction

All samples were confirmed by pathologists. Tumor tissues were stored in formalin-fixed, paraffin-embedded blocks until use. The details of all cytological specimens and tumor tissue preparation were listed in our previous studies.[20,22] RNA was extracted from cytological specimens and tumor tissue using either an RNeasy Mini Kit (Qiagen, Hilden, Germany) or an AmoyDx RNA Kit (Amoy Diagnostics) according to the manufacturer's protocol. The quantity and quality of RNA was subsequently determined on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

ROS1 fusion detection

ROS1 fusion was detected by using an AmoyDx® *ROS1* fusion gene detection kit (Amoy Diagnostics). Detailed methods are provided in our previous studies.[8,9,22] Briefly, mRNA extracted from cytological specimens and tumor tissue was reverse transcribed to cDNA at 42°C, and then amplified by PCR. The RT-PCR conditions were as follows: 95°C for 5 minutes, 15 cycles of denaturation at 95°C for 25 seconds, annealing at 64°C for 20 seconds, and elongation at 72°C for 20 seconds to ensure specificity, and then up to 31 cycles at 93°C for 25 seconds, 60°C for 35 seconds

(data collection), and 72°C for 20 seconds. Patterns of *ROS1* fusion were screened as previously described.[8,9]

Statistical analysis

All statistical analyses were carried out using SPSS version 22.0 (IBM, Armonk, NY, USA). Student's *t*-test was used for comparisons between two different groups, and a *P*-value of <0.05 was considered statistically significant in a two-way analysis.

Results

Summary of specimens acquired

From October 1, 2013 to June 30, 2016, 2,538 patients with advanced NSCLC who received *ROS1* fusion screening by RT-PCR, including 437 (17.2%) with tumor tissue and 2,101 (82.8%) with cytological specimens, were enrolled into our study. A total of 55 (55/2,538, 2.2%) patients were *ROS1* fusion-positive. Of these, 51 (51/55, 92.7%) were further verified by direct sequencing, and no false-positive cases were found. *CD74-ROS1* fusion was found in 21 cases, *EZR-ROS1* fusion in 13 cases, *SLC34A2–ROS1* fusion in nine cases, *SDC4-ROS1* in seven cases, and *GOPC-ROS1* in one case.

Among the 437 patients from whom tumor tissue was collected, 128 patients had paired cytological specimens and were only represented in the tumor tissue group for statistical analyses. Among the 2,101 patients with cytological specimens, 1,648 were collected by needle aspiration, 449 were by effusion samples, and four were by sputum samples. Of the samples collected by needle aspiration, 1,197 were by computed tomography-guided transthoracic needle aspiration (TTNA), 190 were by endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA), 251 were by needle aspiration of superficial lymph nodes (SLN-NA), and ten were by needle aspiration of subcutaneous nodules. Of the effusion samples, 429 were malignant pleural effusions (MPEs), 15 were pericardial effusions, and five were ascites. The types of specimen acquisition methods are also illustrated in Figure 1.

RNA concentrations and *ROS1* fusion detection

In this study, we adopted spectrophotometry to evaluate the RNA concentrations obtained (Table 1). Among the 2,101 cytological specimens, the average, minimum, and maximum RNA concentrations per collection method were 36.13, 0.09, and 593.34 ng/μL for TTNA; 31.93, 0.09, and 485.54 ng/μL for EBUS-TBNA; 34.74, 0.31, and 446.63 ng/μL for SLN-NA; 26.14, 5.30, and 89.60 ng/μL for NA from subcutaneous nodules; 12.51, 5.12, and 20.60 ng/μL for sputum samples; 93.38, 0.60, and 3,768.04 ng/μL for MPE samples; 92.31, 0.64, and 296.06 ng/μL for pericardial effusions; and 76.80, 39.89, and 151.11 ng/μL for ascites. For the 437 tumor tissue samples, the average, minimum, and maximum RNA concentrations were 66.50, 0.14, and 786.62 ng/μL. Across all 2,101 cytological specimens, the average RNA concentration was 47.68 ng/μL (95% CI, 43.24–52.62), which was significantly lower than 66.54 ng/μL (95% CI, 57.18–76.60, $P=0.001$) obtained from the 437 tumor tissue samples (Figure 2). However, the

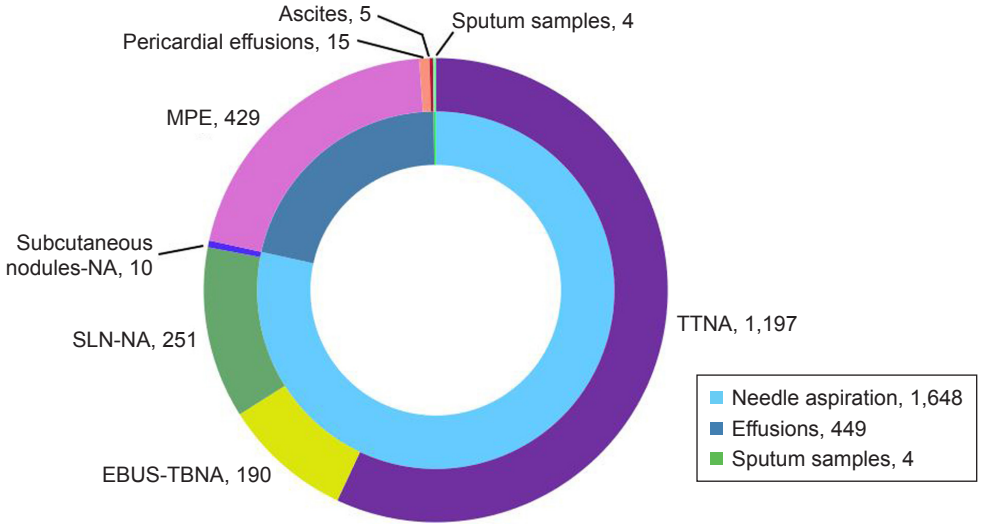

Figure 1 The type of specimens' acquisition.
Abbreviations: TTNA, computed tomography-guided transthoracic needle aspiration; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; SLN-NA, needle aspiration of superficial lymph nodes; subcutaneous nodules-NA, needle aspiration of subcutaneous nodules; MPE, malignant pleural effusion.

Table 1 The RNA yield in the different subgroups of samples

Sample type	Total (n=2,538)		*ROS1*-negative		*ROS1*-positive	
	n	RNA yield* (ng/μL)	n	RNA yield* (ng/μL)	n	RNA yield* (ng/μL)
Tissue	437	66.54 (0.14–786.62)	423	66.58 (0.14–786.62)	14	65.33 (6.37–355.85)
Cytological samples	2,101	47.68 (0.09–3,768.04)	2,060	47.90 (0.09–3,768.04)	41	36.92 (1.59–191.28)
From TTNA	1,197	36.13 (0.09–593.34)	1,180	36.47 (0.09–593.34)	17	13.02 (2.26–74.92)
From EBUS-TBNA	190	31.93 (0.09–485.54)	188	32.14 (0.09–485.54)	2	11.76 (10.69–12.83)
From SLN-NA	251	34.74 (0.31–446.63)	241	35.15 (0.31–446.63)	10	24.87 (3.03–126.78)
From subcutaneous nodules-NA	10	26.14 (5.30–89.60)	10	26.14 (5.30–89.60)		
Sputum samples	4	12.51 (5.12–20.60)	4	12.51 (5.12–20.60)		
MPE	429	93.38 (0.60–3,768.04)	417	93.62 (0.60–3,768.04)	12	85.01 (1.59–191.28)
Pericardial effusions	15	92.31 (0.64–296.06)	15	92.31 (0.64–296.06)		
Ascites	5	76.80 (39.89–151.11)	5	76.80 (39.89–151.11)		

Note: *Data are average concentration (minimum–maximum).
Abbreviations: TTNA, computed tomography-guided transthoracic needle aspiration; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; SLN-NA, needle aspiration of superficial lymph nodes; subcutaneous nodules-NA, needle aspiration of subcutaneous nodules; MPE, malignant pleural effusion.

incidence rates of *ROS1* fusion were similar in both tissue types: 1.95% (41/2,101) in cytological specimens vs 3.20% (14/437, *P*=0.102) in tumor tissues.

Among the 55 *ROS1*-positive patients, 14 had tumor tissue samples and 41 had cytological specimens, including 17 collected by TTNA, two collected by EBUS-TBNA, ten collected by SLN-NA, and 12 collected by MPE (Table 1). For all these *ROS1*-positive patients, the RNA concentrations for specimens ranged from 1.59 ng/μL to 355.85 ng/μL. For 14 *ROS1*-positive patients with tumor tissue, the range of RNA concentrations was from 6.37 ng/μL to 355.85 ng/μL. For 41 *ROS1*-positive patients, RNA concentrations ranged from 1.59 ng/μL to 191.28 ng/μL. The minimum RNA concentration obtained from an MPE specimen was determined, and its fusion status was reconfirmed by direct sequencing as *SLC34A2–ROS1* (E4; E32).

In our study, 128 patients had paired cytological and tissue samples, including four *ROS1* fusion-positive samples. In order to analyze the concordance rate of *ROS1* fusion status between tissue specimens and paired cytological specimens, we chose the only 4 samples with ROS1-positive and 4 with *ROS1*-negative that were randomly selected, all of which were detected by tumor tissue. The consistency with regard to *ROS1* fusion detection between tumor tissue samples and paired cytological samples was 100%.

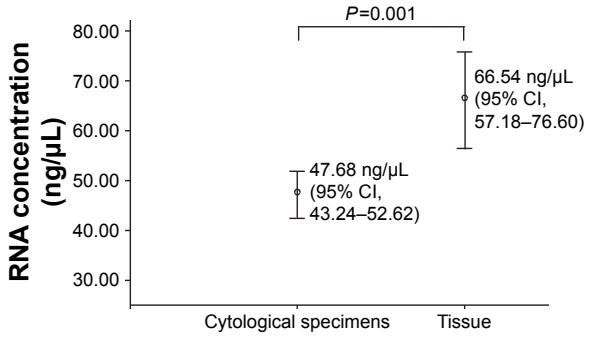

Figure 2 Comparison of average concentrations of RNA between cytological group and tissue group.

The efficacy of crizotinib

Totally, 14 of the 55 patients identified as *ROS1* RT-PCR-positive received the treatment of crizotinib, including eleven with cytological specimens and three with tissue. For eleven patients with cytological specimens, six were female and one had brain metastases. For three patients with tumor tissue, none were female and none had brain metastases. Among the eleven patients with cytological specimens, nine had a partial response and two got a stable disease. Among the three patients with tumor tissue, one showed a complete response and two showed a partial response. Three patients with cytological specimens were lost to follow-up in November 2016. Thus, the objective response rate (ORR) was 81.8% in patients with cytological specimens, similar to the ORR of 100% (*P*=0.604) obtained for the three patients with tumor tissue.

Discussion

To the best of our knowledge, this is the first large-scale retrospective study to comprehensively explore the feasibility of detecting *ROS1* fusion status by RT-PCR from cytological specimens. We compared RNA yields and the incidence rates of *ROS1* fusion between tumor tissue samples from 437 patients and cytological specimens from

2,101 patients. Among the cytological specimens, the average RNA concentration was 47.68 ng/μL (95% CI, 43.24–52.62), which was lower than the average of 66.54 ng/μL (95% CI, 57.18–76.60, *P*=0.001) obtained from tumor tissues. However, no statistically significant difference was observed in *ROS1* fusion incidence between cytological specimens and tumor tissues. In addition, there were no statistically significant differences in ORR with respect to sample type for *ROS1*-positive patients treated with crizotinib. Taken together, these results support the fact that RT-PCR of cytological specimens can be used to detect *ROS1* fusion status in advanced NSCLC patients.

A large retrospective survey of Asian populations indicated that *EGFR* mutation status was detectable using cytological samples in no fewer than 50% of NSCLC patients.[23] Similarly, another study demonstrated that cytological samples can be used successfully for *EGFR* mutation analysis in lung cancer.[24] In addition, we found in a previous study that EBUS-guided needle aspiration can be used to perform molecular analyses for *ERCC1, RRM1*, and *BRCA1*.[25] Similarly, Zhao et al showed that pleural, ascitic, or pericardial effusions of advanced lung adenocarcinoma can be used for detecting *ALK, ROS1*, and *RET* fusion status.[26] Finally, Wang et al indicated a high feasibility for the detection of *ALK* fusion status by RT-PCR from cytological specimens, which might also be considered as a feasible sample source for *ALK* detection in advanced NSCLC patients.[20] Hence, cytological samples may be used for molecular analyses in clinical practice among NSCLC patients.

Till date, no studies have investigated whether *ROS1* fusion testing can be performed in the cytological specimens of advanced NSCLC patients. We found that the RNA yields of cytological specimens were significantly lower than those from tumor tissue. Nevertheless, the incidence rates of *ROS1* fusion were similar (1.95% vs 3.20%, *P*=0.102) between patients with cytological specimens and with tumor tissue. Thus, we can conclude that cytological specimens can be used for *ROS1* fusion detection among patients with advanced NSCLC.

Numerous previous studies have found that FISH, IHC, and RT-PCR can all reliably detect *ROS1* fusion status in advanced NSCLC patients, with concordance rates above 90%.[27–31] Wang et al suggested that the high concordance (99.2%) between FISH and RT-PCR results supports considering RT-PCR as an alternative method for detecting *ALK* fusion status.[32] They also reported that advanced NSCLC patients who are *ALK* fusion-positive, detected by RT-PCR, achieved similar clinical responses to crizotinib compared to those detected by FISH; furthermore, by using RT-PCR approach, two *ALK*-positive patients responded to crizotinib who would otherwise be missed by FISH testing.[32] In a Phase II study of crizotinib, East Asian advanced NSCLC patients had their *ROS1* fusion status assessed through RT-PCR, and the *ROS1*-positive patients who were treated with crizotinib achieved clinically marked benefits and durable responses.[6] Building upon these findings, we adopted the RT-PCR approach for detecting *ROS1* fusion status and found no statistically significant differences in ORR upon crizotinib treatment of patients from the cytological and tissue subgroups.

We must mention that there are several limitations to this study. Firstly, this was a retrospective study, and selection bias was inevitable. The *ROS1* fusion incidence of 2.2% in our study was slightly higher than for several previous reports, which was partially attributable to some patients who were selected from the wild-type *EGFR* and *ALK* population. Secondly, due to the limited number of *ROS1*-positive patients who received crizotinib in our study, the conclusion on its efficacy should be viewed cautiously. Thirdly, more detection methods, such as FISH and IHC, should be adopted to validate our conclusions.

However, taken together, the results of the current study demonstrate that cytological specimens can be used as alternative samples for detecting *ROS1* fusion status by RT-PCR in advanced NSCLC patients.

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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