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ORIGINAL ARTICLE

Significance and evaluation of anaplastic lymphoma kinase by immunohistochemistry in non-small cell lung cancer

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Abstract We used immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR) to evaluate anaplastic lymphoma kinase (ALK) protein expression and gene rearrangements, respectively, in 283 cases of wild-type epidermal growth factor receptor (EGFR) non-small cell lung cancer biopsy specimens. Immunohistochemistry was positive for ALK in 52 cases (18.4 %), and there was no significant difference in staining between various monoclonal antibodies (Roche ALK test kit, D5F3, p-ALK, and EML4-ALK). On RT-PCR, 36 cases (12.7 %) were positive for ALK. Immunohistochemistry and RT-PCR were both positive in 35 cases and both negative in 230 cases, and both have a high consistency (265/283, 93.6 %). Including 17 cases, immunohistochemistry was positive but RT-PCR was negative, and in one case, immunohistochemistry was negative but RT-PCR was positive. On fluorescence in situ hybridization (FISH) testing of these 18 cases, only three cases were positive (one RT-PCR was positive; two immunohistochemistry were positive). There is a high prevalence of ALK positivity in wild-type EGFR non-small cell lung cancer. Immunohistochemistry for the detection of ALK gene rearrangements was highly consistent with RT-PCR, and thus, it is a good screening tool but produces false positive results that necessitate further screening by RT-PCR or FISH.

Keywords Immunohistochemistry · Non-small cell lung cancer · ALK

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Introduction

An echinoderm microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK) fusion gene has long been known to exist in non-small cell lung cancer in 2007 [1], but only in recent years it was found that treatment with crizotinib and other tyrosine kinase inhibitors (TKIs) is effective [2]. Screening patients of ALK gene rearrangements, enabling targeting of treatment, is especially important. The Union for International Cancer Control recommends that, in non-small cell lung cancer, the presence of epidermal growth factor receptor (EGFR) and ALK mutations should be determined [3] to guide treatment. Fluorescence in situ hybridization (FISH) has been the gold standard test for ALK gene rearrangements [4]. However, the EML4-ALK fusion gene is found in only 4–5 % [5] of non-small cell lung cancers, and the use of FISH is especially expensive in all cases. FISH also requires special equipment and strictly controlled laboratory conditions, so it cannot be performed in the most hospitals or even in large general hospitals given the low proportion of patients involved [6]. A simpler and more cost-effective screening method is urgently required.

For detecting ALK gene rearrangements, the results of reverse transcription polymerase chain reaction (RT-PCR) are similar to those of FISH and this method is widely used [7]. The use of immunohistochemical methods to detect EML4-ALK in non-small cell lung cancer was first reported in 2010 [8], and the report has gradually increased since then [9]. However, immunohistochemical findings vary due to the differences of the antibodies used, the sources of specimens, and the criteria and positive control methods employed [10]. The role and significance of immunohistochemistry in screening for EML4-ALK positive need further evaluation in non-small cell lung cancer.

In addition to convenience and economy, immunohistochemical methods have the advantage that both known and

unknown ALK gene changes are detected; they always can be detected in ALK protein levels. However, which antibody is most suitable for this purpose need to be determined [11]. We used various ALK antibodies for immunohistochemical staining of wild-type EGFR non-small cell lung cancer samples and compared the findings with RT-PCR results [12]. Finally, FISH results were used as a gold standard to evaluate the specificity and sensitivity of the immunohistochemical method and RT-PCR method.

Materials and methods

Two hundred and eighty-three cases of wild-type EGFR non-small cell lung cancer were diagnosed on first biopsy at the Clinical College of China Medical University from January 2011 to December 2014. We obtained archived paraffin block specimens from the Department of Pathology. Consent and licensing for the use of specimens were granted by the hospital's Ethics Committee. There were 243 cases of adenocarcinoma and 40 cases of squamous cell carcinoma, 189 cases were male (66.8 %) and 94 cases were female (33.2 %), and the patients were in the 30–84-year age range, with a mean age of 58 years.

Immunohistochemistry

A Roche company ALK immunohistochemical detection kit was used, employing a Roche-automated immunohistochemistry system (model: Ventana) in strict accordance with the manufacturer's instructions [13]. Other antibodies used for immunohistochemistry were D5F3 (1:200; Cell Signaling Technology, Danvers, MA), p-ALK (1:400; Cell Signaling Technology), and EML4-ALK monoclonal antibody (1:200; Cell Signaling Technology). Ethylene diamine tetraacetic acid (EDTA) was used as a buffer (pH = 8), and antigen retrieval at high temperature and pressure 2 min. Staining intensity scoring criteria were as follows: not colored, 0 points; light yellow, 1 point; yellow granules, 2 points; brown granules, 3 points. The percentage of positive cells was scored as follows: <10 %, 0 points; 11–40 %, 1 point; 41–70 %, 2 points; ≥71 %, 3 points. These two scores were multiplied to give the following results: 0–3, negative; ≥4, positive [14]. However, the positive criteria of Roche kit are as follows: positive: brown-stained particles are in the cytoplasm, regardless of the number of its positive cells; negative: not colored [4].

RT-PCR

We used an ALK amplification refractory mutation system (ARMS) detection kit based on Ed biological medicine company (Xiamen, China). RT-PCR method is according to the manufacturer's instructions. The main steps are as follows: Take five tablets of formalin-fixed tissues and add 1 ml of xylene in the centrifuge tube, 1400 rpm for 2 min; After the supernatant was removed, then 1 ml ethanol was added and shook for 10 s. After centrifugation, the supernatant was removed again; RNA was isolated according to the instructions [15]. RNA samples were incubated for 1 h at 42 °C and 5 min at 95 °C and then cooled on ice. The resulting solution was used for PCR amplification. In the amplification process, to avoid evaporation, two or three drops of mineral oil were added and the samples were then placed in a reaction tube at 95 °C for 5 min. The reaction cycle steps were as follows: 95 °C for 30 s for denaturation, 55 °C for 30 s for annealing, and 72 °C for 2 min for extension, for 30 cycles. After the last cycle, the reaction tube was maintained at 72 °C for 5 min to ensure full extension. Carboxy fluorescein (FAM) signal was then detected at 60 °C by real-time PCR and the file was saved. FAM is to detect the amplification of complementary DNA (cDNA) as a reporter gene. An S-shaped FAM amplification curve and a Ct value of less than 30 were considered positive. If the FAM amplification curve was not S-shaped or the Ct value was greater than or equal to 30, the result was considered negative or lower than the kit's detection limit. The RT-PCR results were then compared with the immunohistochemical findings.

FISH

We used an Abbott kit for FISH according to the manufacturer's instructions (Abbott Vysis, Shanghai, China). Positive signal was red and green signal separation prevail (on ×100 magnification, the distance between two positive signals was greater than the maximum radius of a positive signal). We used the following counting method to determine the positivity rate. The first count of 50 cells were positive if positive cells are more than 25 and negative if less than 5; if positive cells are between 5 and 25, then count another 50 cells. At this time, a total of 100 cells were positive if positive cells is greater than 15 and negative if less than 15 [16].

Statistical analysis

All statistical analyses were performed using SPSS 17 (IBM, Armonk, NY). Immunohistochemistry results and RT-PCR results were analyzed using the sensitivity and specificity regarding the FISH results as a gold standard [17]. Two-tailed P values of <0.05 were considered statistically significant.

Results

Immunohistochemistry

Among 283 cases of wild-type EGFR non-small cell lung cancer, ALK-positive in 52 (18.4 %) cases were shown by immunohistochemistry and microscope observations showed

the vast majority of cell are diffused, brown-stained particles mainly inside the cytoplasm. Only 18 cases (16 cases of adenocarcinoma and 2 cases of squamous cell carcinoma) were weakly positive (the Roche detection kit showed fewer positive cells), and there were 231 ALK negative cases (81.6 %). The positivity rates of the four antibodies used were almost the same, except for the higher background staining with the EML4-ALK antibody; the other three antibodies did not differ significantly. However, with the Roche kit, ALK detection is "all or nothing"; so determining whether a sample was positive or negative was easier with this method (Fig. 1).

RT-PCR

The positive results of RT-PCR were 36 (12.7 %) cases and 247 (87.3 %) cases were negative for ALK. Immunohistochemical results and RT-PCR results were consistent in 265 (93.6 %) cases, including 35 positives (Fig. 2) and 230 negatives. The sensitivity of immunohistochemistry was 97.3 % (37/38), and the specificity was 93.9 % (230/245). The sensitivity of RT-PCR was 94.5 % (37/39), and the specificity was 100 % (230/230). The comparison results of the sensitivity and specificity are shown in Table 1.

FISH

We used FISH as the gold standard method in 18 cases in which the immunohistochemical and RT-PCR results were inconsistent. Only three cases were found to be positive on FISH. In two cases, immunohistochemistry was weakly positive and RT-PCR was negative; in one case, RT-PCR was positive and immunohistochemistry was negative (Fig. 3). Fifteen cases were found to be negative on FISH; all of these cases were positive on immunohistochemistry and negative on RT-PCR. The false positive rate was 28.8 % (15/52). Immunohistochemistry had a higher false positive rate, but the false negative rate with this method was only 0.40 % (1/247). Given that immunohistochemistry methods are less expensive and easy to perform, this may be considered an ideal screening tool for ALK protein expression in non-small cell lung cancer, particularly in primary hospitals.

Discussion

Although the EML4-ALK fusion gene accounts for only a small proportion of non-small cell lung cancers (about 5 %),

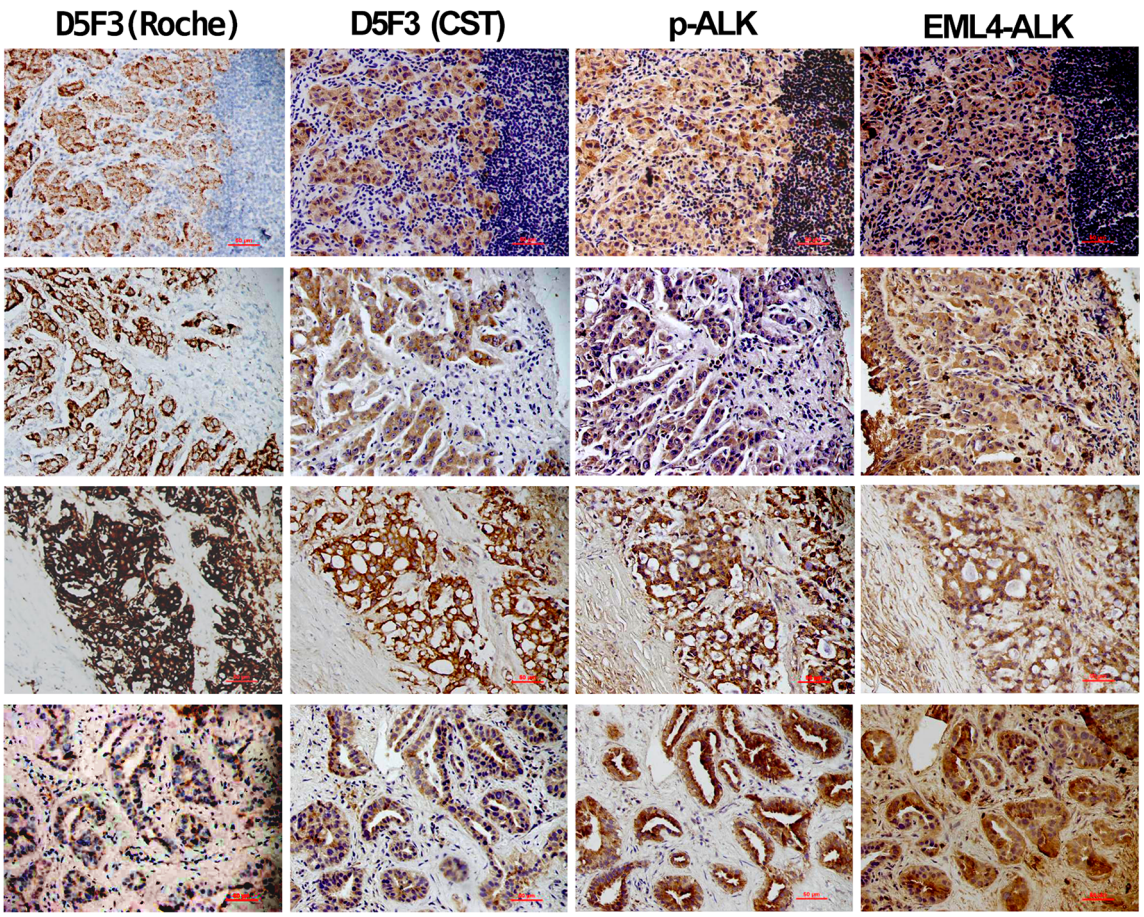

Fig. 1 Serial sections of the same specimen comparing immunohistochemistry with different antibodies

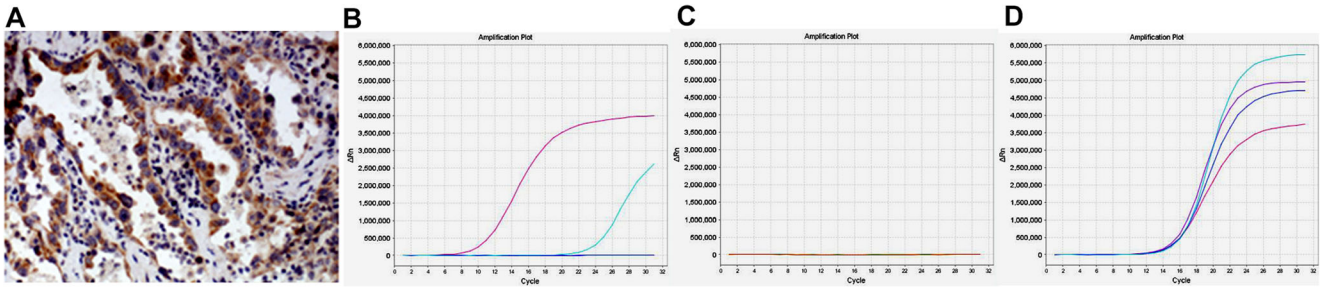

Fig. 2 Immunohistochemistry and PCR results. **a** Immunohistochemical staining with D5F3 antibody was strongly positive. **b** In the same case, RT-PCR (ARMS) was also positive. **c** RT-PCR of a negative control. **d** RT-PCR of a positive control

EGFR mutations occur at a higher rate (40 %) [18]. The EML4-ALK fusion gene has been shown to be significantly enriched in wild-type EGFR non-small cell lung cancer from about 5 to 12–20 % [19, 20]. In the present study, the prevalence was 13.42 % (38/283) (RT-PCR-positive in 36 cases and FISH-positive in two cases), which is similar to the findings reported in the literature. The presence of ALK mutant enrichment in wild-type EGFR lung cancer cases, which is relevant to the genetic testing of non-small cell lung cancer patients, enable targeted therapy with TKIs.

In this study, ALK mutation positive rate is 18 % by immunohistochemical methods. Although it is higher than the positive rate of RT-PCR (13 %), there is only one case of false negative cases after the FISH method validation. Nevertheless, the immunohistochemistry method remains a screening method; the positive cases still need further validation in other ways with the conditions of the laboratory. After all, targeted therapies are more expensive and have side effects. Because immunohistochemical method is simple, economical, and fast, it does not require strict environmental, equipment, and more technical content; it can carry out the inspection work even at the grassroot-level hospitals [21]. Therefore, immunohistochemical method is expected to become one of the methods for screening ALK mutations. Compared with RT-PCR, immunohistochemical method is to detect the expression of protein, no matter how the ALK gene changes; as long as it has changed in the protein expression, it can be detected. Due to the emergence of a variety of EML4-ALK gene mutation, so the probe of RT-PCR method also needs to continue to increase; therefore, the RT-PCR method also has limitations. In this study, two cases were found to be positive by immunohistochemistry method, but the result of RT-PCR was negative, and the final FISH was proved to be ALK-positive.

Of course, the immunohistochemical method also has some limitations for the detection of ALK mutation; the most obvious is the emergence of a higher false positive rate. Any non-standard operation may result in a false positive or false negative from the tissue to the dyeing in the whole process. The cause of high false positive in this study may be related to the excessive time to repair the antigen. For example, the application of different antibodies, and the same antigen repair conditions, results in non-specific staining and thus was judged to be positive. On this point, we need to further verify. In addition, immunohistochemistry is only to detect the protein expression, but it cannot reflect the changes in the EML4-ALK gene; therefore, it is still insufficient to guide and understand the significance of targeted therapy.

At present, it is generally believed that the FISH method is the gold standard for the detection of the EML4-ALK gene mutation in the lung cancer. Due to the incidence of EML4-ALK, mutation rate is low in the non-small cell lung cancer (NSCLC); if all quasi test cases were detected by the FISH method, it will not only need more time and consuming; the results can only be obtained a few positive cases. This is exactly why we did not use the FISH method to verify all the cases; we only regard the FISH method as a gold standard of evaluation indicators to assess the results of immunohistochemistry and RT-PCR.

Comparing the three methods of immunohistochemistry, RT-PCR, and FISH to detect the ALK mutations, the literature indicates that RT-PCR has a high sensitivity and specificity in detecting EML4-ALK mutations, which can be applied to a variety of samples and can detect a variety of EML4-ALK mutants [22]. But RT-PCR is still an inadequate initial test for detecting ALK positive, due to the specificity of the probe; there will be a high false negative, which indicates that the

Table 1 Immunohistochemical results and RT-PCR results compared with FISH

FISH results	Immunohistochemical results		Total	RT-PCR results		Total
	Positive	Negative		Positive	Negative	
Positive	37	1	38	36	2	38
Negative	15	230	245	0	245	245
Total	52	231	283	36	247	283

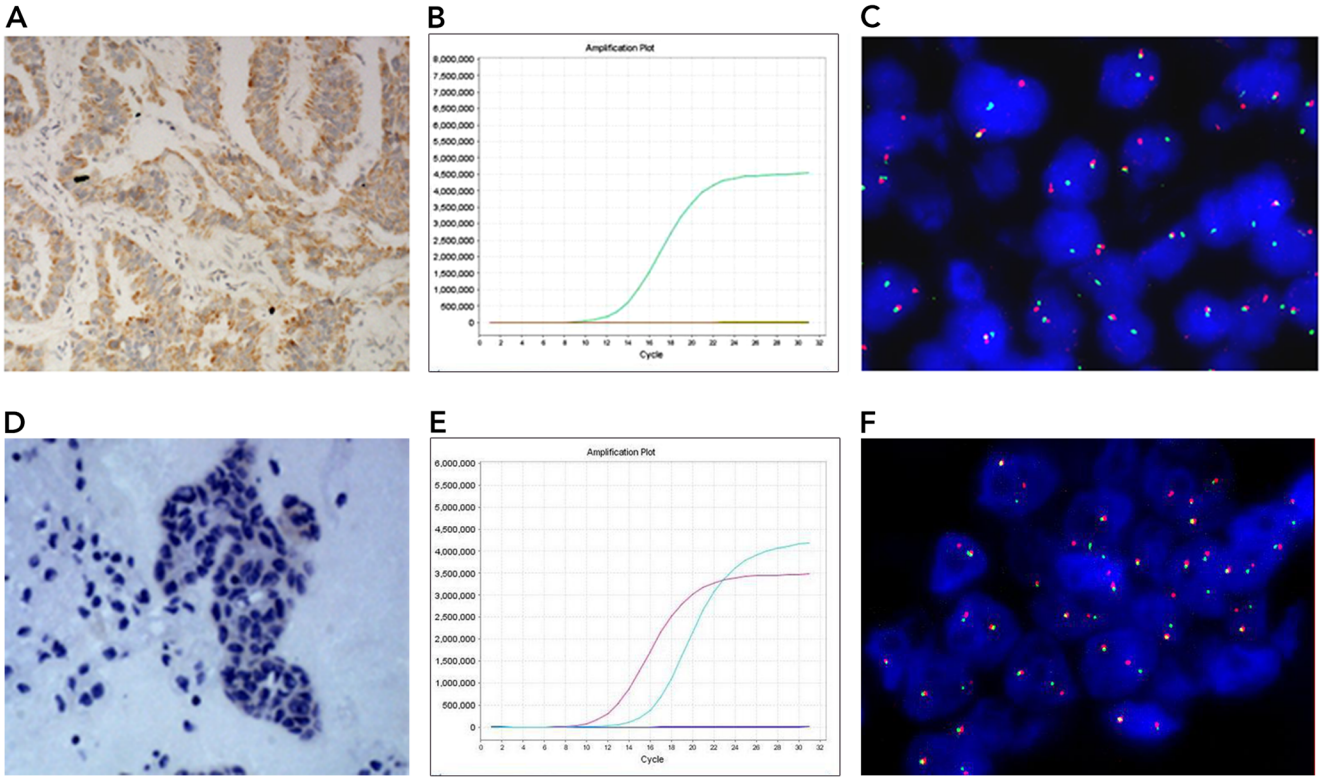

Fig. 3 Immunohistochemistry and PCR results contrast with FISH. **a** Immunohistochemistry was weakly positive. **b** The same sample was negative on ARMS. **c** FISH verified the positive result by the separation of *red* and *green* signals. **d** Immunohistochemistry was negative. **e** The same case was positive on ARMS. **f** FISH verified the positive result by the separation of *red* and *green* signals

immunohistochemistry method is a more effective screening tool [23]. And the immunohistochemical results compared with a new generation of sequencing (NGS) has been reported, indicating that FISH method of detecting EML4-ALK rearrangement may miss a significant number of patients who could benefit from targeted ALK therapy; so the IHC method should be strongly considered [24].

In conclusion, immunohistochemical method is one of the effective methods for the detection of EML4-ALK gene changes; it will get a higher positive rate especially for the

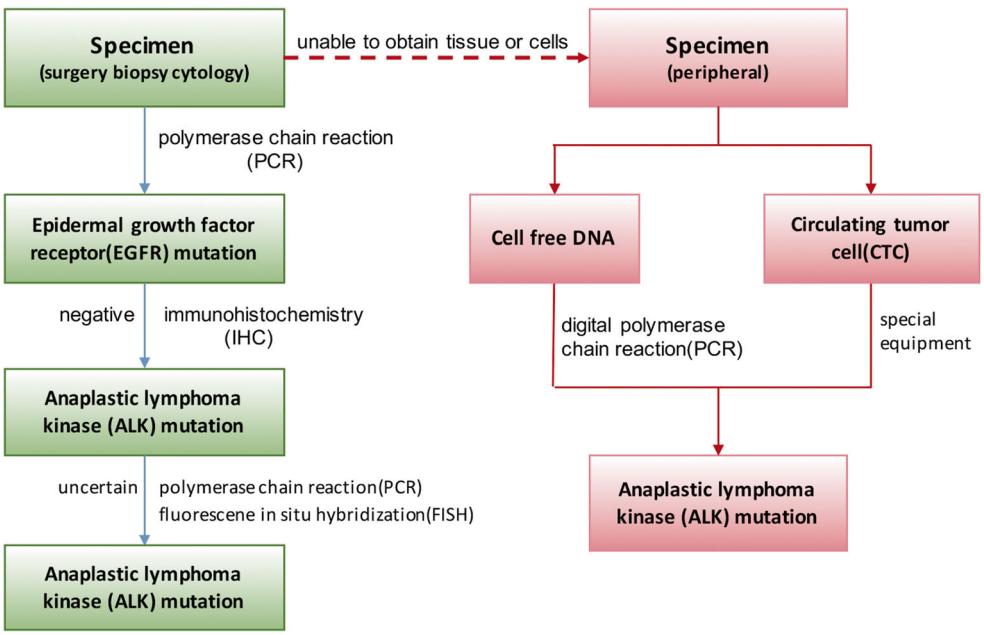

Fig. 4 Immunohistochemical methods can be used to screen for ALK changes in non-small cell lung cancer. It is unclear which other method should be used for verification, ALK can also be detected in their wild-type cases, after the detection of EGFR, obtain higher positive results

detection of wild-type EGFR non-small cell lung cancer. But the immunohistochemical method is just a screening method; it may have a higher false positive. So it is also necessary to use other methods for further verification. In addition, we found that the results with the Roche ALK test kit were similar to those obtained using D5F3 and p-ALK monoclonal antibodies [25]; thus, the choice of antibody is a matter of preference and habit. We propose EML4-ALK determination as a reference for practical work (Fig. 4).

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Compliance with ethical standard

Conflicts of interest None.

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