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Letter to the Editor

Reply to Lambros *et al*

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To the Editor: We appreciate the comments of Lambros *et al*[1] about our study that reported coexistent genetic alterations of *ALK*, *RET*, *ROS1*, and/or *MET* in 15 cases of lung adenocarcinoma.[2] It is gratifying that Lambros *et al* agree with our conclusion that the co-existences of two or more driver mutations are rare but true events in lung adenocarcinoma, although these mutations are historically considered as mutually exclusive. In regard to their concerns about the cutoff values for *ROS1* and *RET* fluorescent *in situ* hybridization (FISH) tests in our study, the *ROS1* and *RET* FISH tests we report were developed and validated in our clinical diagnostic laboratory by following the American College of Medical Genetics (ACMG) technical standards and guidelines for FISH tests,[3,4] since there is currently no FDA-approved test/kit for either *ROS1* or *RET* rearrangement available yet. As pointed by Lambros *et al*, the normal cutoff values for our tests are lower than that reported by other research groups.[5–7] This is most likely attributable to different statistical methods to generate cutoff values for each abnormal FISH signal pattern in these studies. The confidence interval around the mean (binomial distribution) method was employed in our study. This method usually produce stringent (low) cutoff values, which in turn benefit patients with a truly positive but low-level biomarker to be diagnosed and facilitating their eligibility for a therapeutic intervention (eg, a clinical trial). Presumably, the inverse beta function method that usually produces conservative (high) cutoff values has been utilized in at least a subset of other studies.[3,4] Although the high cutoff values usually yield very few false-positive results, the possibility of false-negative results is simultaneously increased. As stated by Shan *et al*,[7] with a cutoff value of ≥15% for *ROS1* FISH test, 3 of 16 cases with documented *ROS1* rearrangement confirmed by both ROS1-immunohistochemistry (IHC) and quantitative real-time reverse transcription-PCR (qRT-PCR) were misdiagnosed as negative by FISH. Similar observations have been made for *RET* FISH testing as well.[8] Both the *ROS1* and *RET* FISH tests have been re-validated more than twice and calibrated periodically by following the ACMG standards and guidelines in our laboratory.[3,4] A slight modification of the cut-off values was performed after each re-validation/calibration, but no change of any previous test results for these two biomarkers has been caused consequently. In fact, all cases included in our report[2] have demonstrated an apparent positive result for *RET* and/or *ROS1* rearrangement (eg, positive cells ≥15%; Table 1). We agree with Lambros *et al* that a second tier of test(s) such as immunohistochemistry (IHC), qRT-PCR and/or next-generation sequencing (NGS) can be applied to further confirm a positive FISH result, especially for a borderline result, although sometimes results obtained by FISH, IHC, qRT-PCR, and/or NGS may not be concordant.[1,5–8]

Lambros *et al* also expressed concern about the high detection rate of triple or even more mutations in the same cases in our study. In addition to coexistent mutations involving *ALK*, *RET*, *ROS1*, or *MET*, 10 of 15 cases also exhibited mutations of *TP53* ($n=6$), *EGFR* ($n=5$), *KRAS* ($n=3$), and rarely other genes. We believe that this detection rate is mainly attributable to the utilization of a NGS panel of genes in our study. The *EGFR* and *KRAS* mutations were confirmed by Sanger sequencing subsequently, and all 5 patients (cases 1 and 12–15) in our study had received or are receiving EGFR inhibitor treatment.[2] Michels *et al*[9] reported that 10 of 22 European lung cancer patients with *RET*-rearrangement also exhibited additional genetic aberrations, 7 with *TP53* mutation, 1 with *CTNNB* mutation, 1 with low level *MET* amplification, and 1 with both *TP53* and *EGFR* mutations. In their study, an NGS of a panel of 14 genes and Sanger sequencing of selected exons of four genes (*EGFR*, *KRAS*, *HER2*, and *BRAF*) were performed in 18 and 4 cases, respectively.

Therefore, simultaneously testing multiple biomarkers and utilization of the advancing technologies,

Table 1 Detailed FISH results in 15 cases with coexistent genetic alterations of *ALK*, *RET*, *ROS1* and *MET*

Case No.	*Detailed FISH results*
1	*ALK*+ (20%); *MET*+ (average *MET* copy number/cell = 6)
2	*ALK*+ (29%); *MET*+ (average *MET* copy number/cell = 6)
3	*ALK*+ (46%); *MET*+ (*MET*:*CC7* = 2.1; average *MET* copy number/cell = 13.2)
4	*ALK*+ (78%); *RET*+ (51%)
5	*ALK*+ (50%); *ROS1*+ (51%)
6	*ALK*+ (26%); *ROS1*+ (15%)
7	*ALK*+ (17%); *ROS1*+ (27%)
8	*ALK*+ (22%); *ROS1*+ (25%)
9	*RET*+ (50.5%); *MET*+ (average *MET* copy number/cell = 7.2)
10	*RET*+ (50%); *MET*+ (average *MET* copy number/cell = 7)
11	*RET*+ (55%); *MET*+ (average *MET* copy number/cell = 5.1; cluster signal in >10% cells)
12	*RET*+ (34.5%); *MET*+ (average *MET* copy number/cell = 6.5; cluster signals in >10% cells)
13	*RET*+ (20%); *ROS1*+ (27%)
14	*RET*+ (27%); *ROS1*+ (17%)
15	*ROS1*+ (18.5%); *MET*+ (*MET*:*CC7* = 2.35, average *MET* copy number/cell = 9.9)

such as the NGS, will likely reveal the coexisting genetic aberrations in more lung cancer cases. On the other hand, the frequencies in all mutations detected in the same case may or may not be the same. Therefore, the coexisting genetic aberrations may or may not present in the same tumor cells, indicating the complexity of tumor heterogeneity. We very much appreciate the thoughtful letter of Lambros *et al* and this opportunity to respond.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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