Correspondence

EML4-ALK Fusion in Lung

To the Editor-in-Chief:

The recent article by Martelli et al¹ reports (i) the detection of *EML4-ALK* fusion cDNA² not only in non-small cell lung cancer (NSCLC) specimens but in non-tumor lung tissues, (ii) a very low proportion of FISH-positive cells for *ALK* rearrangements among *EML4-ALK*-positive specimens, and (iii) the failure to detect *EML4-ALK* protein by immunohistochemistry (IHC) and Western blotting. Based on these lines of observation, the authors questioned the clinical relevance of *EML4-ALK* in the carcinogenesis of NSCLC.

Although detection of fusion kinases in normal tissues is a potentially interesting observation, caution is warranted in the interpretation of their results.^{1,3} They replicated thrice the reverse transcription-polymerase chain reaction (RT-PCR) for *EML4-ALK* and noted that "In half of the (positive) cases, one replicate experiment did not confirm the fusion transcript was present." They then suggested that the fusion gene was "expressed at very low level." It is, however, also quite possible that such unstable PCR results may simply represent contaminated experiments. If this is the case, a discussion on FISH and protein analyses would become irrelevant. In their report, the presence of the *EML4-ALK* fusion gene was only evidenced by unstable RT-PCR results and a small proportion of FISH-positive cells among specimens.

In this regard, it was surprising that the authors had not tried genomic PCR to exclude the possibility of PCR contamination.^{1,3} In most of their fusion-positive cases, they found the *EML4-ALK* variant 1 cDNA, in which exon 13 of *EML4* cDNA is connected to exon 20 of *ALK* cDNA. Because the length of intron 14 of *EML4* gene and intron 19 of *ALK* gene is 5724 bp and 1932 bp, respectively, the maximum size of the genomic PCR to detect the gene fusion should be ≈7.7 kbp, which is within the scope of current long-range PCR systems. Indeed, we have been able to detect genomic PCR products among >50% of the fusion cDNA-positive cases. Interestingly, the break/fusion points in the genome vary substantially among NSCLC specimens,^{2,4,5} and we have not obtained, to date, any pairs of NSCLC specimens carrying identical break/fusion points in their genome (even among those positive for the same *EML4-ALK* variants).

We speculate, therefore, that (i) if none of the fusion cDNA-positive cases reported by Martelli et al^{1,3} produce specific genomic PCR products, then the fusion cDNA products likely arose from cDNA-contamination, (ii) if the

fusion cDNA-positive cases yield identical genomic PCR products, then the fusion cDNAs likely arose from specimen-contamination, and (iii) if the fusion cDNA-positive cases display distinct genomic fusion points, then each specimen was truly positive for the *EML4-ALK* fusion gene. Without such careful examination, we have to conclude that their claims in the article have not as yet been clearly demonstrated.

As described previously,⁶ immunohistochemical detection of the *EML4-ALK* protein is highly difficult, probably owing to the weak activity of the *EML4* promoter that drives the expression of *EML4-ALK* messages. We have thus examined the suitability of commercially available antibodies to *ALK* for IHC and successfully developed the intercalated antibody-enhanced polymer (iAEP) method, which enables reliable detection of *EML4-ALK* among formalin-fixed and paraffin-embedded specimens.⁶ The same specimen positive for *EML4-ALK* RT-PCR can be, for instance, readily stained to be positive with iAEP, but negative with conventional IHC methods (see Supplemental Figure S1 in Ref. 6). We thus agree with Martelli et al that screening of NSCLC specimens with conventional IHC methods will not detect *EML4-ALK* protein, but strongly argue that such failure does not simply indicate the absence of *EML4-ALK*. For such screening, we recommend iAEP or other sensitive techniques.⁷

It should be further noted that, in both our⁶ and other researchers' IHC analyses,⁷ almost all tumor cells in a given *EML4-ALK*-positive specimen were positively immunostained with anti-*ALK* antibodies, suggesting a homogenous presence of *EML4-ALK* within a tumor. Such observation is, however, in contrast to the FISH data by Martelli et al, which show that the *ALK* rearrangement was only positive in ≈2% of tumor cells in a given *EML4-ALK*-positive specimen. On the contrary, FISH analyses of our *EML4-ALK*-positive samples clearly demonstrate that most of the tumor cells harbor rearranged *ALK* alleles, implying that the generation of the *EML4-ALK* fusion gene is an early event in NSCLC carcinogenesis. The homogenous presence of *EML4-ALK* in our fusion-positive tumors, as demonstrated by both FISH and IHC, further raises a concern about the "EML4-ALK-positive tumors" as defined by Martelli et al.

Specific inhibitors to *ALK* enzymatic activity are already in clinical trial, as reported at the 2009 annual meeting of American Society of Clinical Oncology and the European Cancer Organization and Congress of the European Society for Medical Oncology.⁸ Such reports reveal only

modest and transient side effects (nausea, vomiting, and diarrhea) with their ALK inhibitor, but without severe damage in hematopoiesis or renal function. On the other hand, the marked therapeutic efficacy of their compound against EML4-ALK-positive NSCLC makes it one of the rare, highly successful molecular targeted therapies against human cancer, in line with imatinib mesylate and gefitinib/erlotinib. These data further reinforce the essential role of EML4-ALK in the carcinogenesis of NSCLC, and question the validity of the conclusions led by Martelli et al.^{1,3}

Hiroyuki Mano

*The University of Tokyo
Tokyo, Japan*

Kengo Takeuchi

*The Cancer Institute
Tokyo, Japan*

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Authors' reply:

In their letter, Mano and Takeuchi claim that our unstable PCR results in normal and cancerous lung tissues could be attributable to contamination. However, as clearly illustrated in our article,¹ serial dilution experiments in the H2228 cell line demonstrate the specificity and sensitivity of our RT-

PCR assay. Furthermore, the identification in our EML4-ALK fusion positive tissues of alternative isoforms of variant 3, rather than the described two isoforms coexpressed in the H2228 cell line, is indicative of exclusive events in tumors, making contamination unlikely. Lastly, our experiments were confirmed independently in two laboratories (Milan and Barcelona) and always contained appropriate negative PCR controls.

We disagree with Mano et al's claim that the results of genomic PCR could be used to prove a possible RT-PCR contamination in our samples, which can only be excluded by the use of appropriate controls and procedures, as outlined above. However, we used genomic PCR to amplify the sequence flanking the *EML4-ALK* variant 1 breakpoint in four positive NSCLC samples. Even though a strong amplification product had been obtained from the same DNA templates using primer sets amplifying a control genomic locus of similar size to that of the cases so far reported in literature, no amplification of the *EML4-ALK* variant 1 fusion product was identified, suggesting only a minority of cells carried the *EML4-ALK* gene. These findings concur with Maes et al² who reported that, in lymphoid tissues, high level detection of *NPM-ALK* and *AT1C-ALK* fusion transcripts coincided with *ALK* gene rearrangements (as detected by cytogenetics and FISH), whereas low-level detection was not supported by genomic evidence of rearrangements.

In our article,¹ we clearly stated that, unlike observations in ALK+ lymphomas, tumor cells from NSCLC specimens expressed such a low amount of the EML4-ALK fusion protein that immunoprecipitation and immunohistochemistry performed with the commercially available antibodies are unable to detect it. This is in keeping with the observation that the EML4-ALK fusion protein is detectable only using highly sensitive methods, such as mass spectrometry³ or the intercalated antibody-enhanced polymer (iAEP) method⁴ which, unfortunately, are not available in all pathology laboratories and are difficult to standardize. Therefore, the question of how best to detect the EML4-ALK fusion protein remains unanswered.

Issues concerning the frequency, heterogeneity, and tissue specificity of the *EML4-ALK* rearrangement must also be addressed carefully.

Frequency

We recently extended our FISH analysis to 173 surgically resected lung cancer specimens (mainly adenocarcinoma) from an unselected group of Caucasian patients. The incidence of truly positive cases (>50% FISH positive, fusion transcript, and protein positive) was only 0.6% (1/173 cases), which reinforces the results in our article and is in keeping with Rodig et al's⁵ recent report of 1/227 (0.45%) ALK rearranged case in a series of surgically treated Western adenocarcinoma.

Heterogeneity

The heterogeneity of the *EML4-ALK* rearrangement we detected by FISH was confirmed by others in primary tumors and cell lines^{6,7} and is supported by functional studies showing that the magnitude of growth inhibition by siRNA-

mediated silencing did not correlate with the number of cells harboring the rearrangement and the lack of growth inhibition in 50% of *EML4-ALK*-positive cell lines. These observations suggest that additional signaling mechanisms independent of ALK may regulate growth and cell proliferation.

Specificity

Claims from Mano's group that the *EML4-ALK* product is specific for NSCLC is contradicted by our findings in normal tissues^{1,8} and by a recent study from Lin E. et al,⁶ who found *EML4-ALK* fusions in breast (2.4%) and colorectal (2.4%) cancer, in addition to NSCLC.

Finally, we wonder whether it is really appropriate to compare treatments such as ALK inhibitors in NSCLC with imatinib mesylate and gefitinib/erlotinib in other human neoplasms. In fact: i) the role of *EML4-ALK* in NSCLC is not as well established as that of BCR/ABL in chronic myeloid leukemia (CML); ii) NSCLC responses to ALK inhibitors⁹ are not as remarkable as the CML response to imatinib mesylate; and iii) patients with NSCLC were treated with a multitikinase, c-MET and ALK, inhibitor.⁹ Considering that about 20% of NSCLC have MET amplification and overexpression and that MET rearrangements are homogeneous in lung cancer,¹⁰ it may be possible that responses to the multitikinase inhibitor may be related to other coexisting oncogenic events, independently of ALK.

In conclusion, although we fully acknowledge the importance of Soda et al's discovery,¹¹ we believe that additional studies are required to elucidate the concurrent genetic events and cellular settings necessary for *EML4-ALK* to exert an oncogenic function and to better define the role of *EML4-ALK* in diagnosis and targeted therapy of NSCLC.

Brunangelo Falini
 Maria Paola Martelli

University of Perugia
 Perugia, Italy

Stefano A. Pileri

University of Bologna
 Bologna, Italy

Gabriella Sozzi
 Patrizia Gasparini

Istituto Nazionale dei Tumori
 Milan, Italy

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