BRIEF REPORT

MET Exon 14 Alterations and New Resistance Mutations to Tyrosine Kinase Inhibitors: Risk of Inadequate Detection with Current Amplicon-Based NGS Panels

Brigitte Poirot, PharmD,[a,b,c] Ludovic Doucet, MD,[d] Shirine Benhenda, PhD,[c] Jérôme Champ, MS,[a] Véronique Meignin, MD, PhD,[e] Jacqueline Lehmann-Che, PharmD, PhD[a,b,c,*]

[a]*Molecular Oncology Unit, Saint-Louis Hospital, Assistance Publique Hôpitaux de Paris, Paris, France*
[b]*Paris Diderot University, Sorbonne Paris Cité Paris, France*
[c]*INSERM U944, Saint-Louis Hospital Paris, France*
[d]*Department of Medical Oncology, Saint-Louis Hospital, Assistance Publique Hôpitaux de Paris, Paris, France*
[e]*Department of Pathology, Saint-Louis Hospital, Assistance Publique Hôpitaux de Paris, Paris, France*

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ABSTRACT

Introduction: Targeted therapies such as tyrosine kinase inhibitors (TKIs) have dramatically improved the treatment of lung adenocarcinoma, and detection of activating mutations of genes such as *EGFR* or anaplastic lymphoma kinase gene (*ALK*) is now mandatory in the clinical setting. However, additional targetable alterations are continuously being described and forcing us to adapt our detection methods. Here we have evaluated the ability of eight amplicon-based next-generation sequencing (NGS) panels to detect the recently described mesenchymal epithelial transition factor (*MET*) exon 14 (*MET*ex14) alterations or new mutations conferring resistance to TKIs.

Methods: A total of 191 tumor samples from patients with NSCLC were screened for *MET*ex14 mutations by Sanger sequencing, and 62 additional cases were screened by Sanger sequencing and two amplicon-based NGS panels. *In silico* comparison of eight commercially available targeted NGS panels was also performed for the detection of *MET*ex14 alterations or *ALK*, *ROS1,* or *EGFR* resistance mutations.

Results: NGS analysis of the positive *MET*ex14 cases revealed a false-negative case because of amplicon design. Moreover, *in silico* analysis revealed that none of the eight panels considered would be able to detect more than 63% of literature-reported cases of *MET*ex14 mutations and similar limitations would be expected with new *ALK*, *ROS1,* or *EGFR* resistance mutations.

Conclusions: We have illustrated major limitations of commercially available amplicon-based DNA NGS panels for detection of *MET*ex14 and recently described resistance mutations to TKIs. Documented choice of available panels and their frequent reevaluation are mandatory to deliver the most accurate data to the clinician for therapeutic decisions.

Keywords: Non–small cell lung cancer; Molecular testing; Targeted therapies; NGS cancer panels

Introduction

The constant discovery of targetable activating mutations and new-generation tyrosine kinase inhibitors (TKIs) has improved response rates, time to progression, and overall survival in advanced NSCLC. Clinical studies have recently shown that in addition to *EGFR*-mutated or anaplastic lymphoma kinase gene (*ALK*)-mutated tumors sensitive to TKIs, tumors with mesenchymal epithelial transition factor (*MET*) exon 14 (*MET*ex14) splicing mutations or mutations altering a direct binding site for c-Cbl

***Corresponding author.**

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Address for correspondence: Jacqueline Lehmann-Che, PharmD, PhD, Laboratoire d'Oncologie Moléculaire, Hôpital Saint-Louis, 1 av Claude Vellefaux, 75 010 Paris, France. E-mail: jacqueline.lehmann-che@aphp.fr

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at tyrosine 1003 (Tyr1003) could be sensitive to *MET* inhibitors.[1] Furthermore, mutations conferring acquired resistance to EGFR, ALK, or ROS1 inhibitors are emerging and have to be screened to adjust the treatment of tumors with the corresponding alterations.[2–6] These new mutations must be detected in diagnostic tests as efficiently as are other well-known targetable mutations, such as *EGFR* activating mutations. This constantly growing number of essential biomarkers makes it necessary to evolve from single-gene companion tests to next-generation sequencing (NGS). In a clinical context, NGS targeted gene panels represent a good option for rapid, cost-efficient, and sample-preserving cancer genomic profiling to guide therapeutic decisions, but this approach could present some drawbacks that are illustrated here.[7,8]

In this article, we have reported our experience in detecting *MET*ex14 mutations by Sanger sequencing with homemade design versus by two commercially available amplicon-based NGS panels. We highlight NGS panel limitations concerning detection of *MET* mutations, in particular, false-negative cases. We therefore analyze *in silico* the ability of several other amplicon-based NGS panels to specifically detect *MET*ex14 mutations as well as the new resistance mutations of *ALK*, *ROS1,* or *EGFR*. Our results reveal that currently, these panels are not tailored to detect all these recently described mutations. It seems necessary to use additional or alternative solutions to decrease the risk of false-negative results and inappropriate patient selection for targeted therapies in NSCLC.

Materials and Methods

Patients and Samples

On the basis of tumor samples from patients with NSCLC referred to our laboratory for molecular characterization between October 2011 and August 2016, 253 tumors with a tumor content higher than 20% and without *EGFR* (exons 18 to 21), *KRAS* (exon 2), or *BRAF* (codon Val600) activating mutation were screened for *MET*ex14 splicing or Tyr1003 mutations.

MET*ex14 Mutation Detection*

Sanger sequencing was performed after polymerase chain reaction amplification of two overlapping amplicons, exploring the nucleotides c.2888-65 to c.3003 and c.2939 to c.3028+45 (NM_000245). This assay is designed to cover more than 95% of *MET*ex14-mutated cases described by Frampton et al.[9] (Supplementary Table 1). Purified DNA was sequenced using a BigDye Terminator Cycle Sequencing Kit on a 24-capillary ABI3500 DNA Analyzer, and sequence interpretations were performed with SeqScape software (Thermo Fisher Scientific, Waltham, MA). All mutations were confirmed in a second independent experiment.

Because of implementation of NGS in the laboratory, the 57 samples received after January 1, 2016, were also prospectively analyzed by targeted NGS technology using the TruSight Tumor 26 (TS26) on a MiSeq sequencer (Illumina, San Diego, CA) or Ion AmpliSeq Colon and Lung Research Panel v2 (CLV2) on an Ion PGM sequencer (Thermo Fisher Scientific). Bioinformatic analysis was performed with Sophia Data-Driven Medicine software as a service (Sophia Genetics, Saint-Sulpice, Switzerland).

In Silico *Comparison of Commercially Available DNA Amplicon-Based NGS Panels*

For *in silico* comparison, we selected eight amplicon-based, largely commercially available NGS kits (TruSight Tumor 15, TruSight Tumor 26, TruSeq Amplicon Cancer Panel [Illumina], Ion Ampliseq Colon and Lung Cancer Research Panel v2, Ion Ampliseq Cancer Hotspot Panel [Thermo Fisher Scientific], Tumor Hotspot MASTR Plus [Multiplicom, Niel, Belgium], ThunderBolts Cancer Panel [Raindance Technologies, Billerica, MA], and Accel-Amplicon 56G Oncology Panel v2 [Swift Biosciences, Ann Arbor, MI]) for DNA analysis designed to explore hotspot mutations useful notably in the lung cancer context. Primer location was used to determine the panels' ability to detect *in silico* relevant *MET*ex14 and *ALK*, *ROS1,* or *EGFR* C797S resistance mutations. *MET*ex14 mutations reported only in patients with lung cancer were extracted from Frampton et al.[9] (Supplementary Data), Awad et al.,[10] Liu et al.,[11] Paik et al.,[12] and Tong et al.[13] or identified in our laboratory. Major *ALK* (NM_004304), *ROS1* (NM_002944), and *EGFR* C797S (NM_005228) resistance mutations were also investigated.[2–6] Alterations located entirely inside an amplicon (primers excluded) were considered potentially detectable irrespective of their size.

Results

MET*ex14 Detection by Sanger Sequencing*

A total of 253 samples from 250 patients with NSCLC (190 men and 60 women) were tested for *MET*ex14 mutations. The median age of the patients was 62 years (range 25–97). The cohort of 253 samples was composed of 191 samples from patients with adenocarcinoma, 24 from patients with squamous cell carcinoma, and 38 from patients with NSCLC not otherwise specified. Sanger sequencing was interpretable for 246 of 253 samples. *MET*ex14 alterations were identified in five patients (2%) listed in Table 1. Identical or similar mutations were previously reported in the literature. As described in other studies, patients presenting *MET*ex14 mutation tended to be older,[10,13,14] even if our positive cohort was too small to allow statistical analysis.

Table 1. Clinical and Pathological Characteristics of Patients with *MET* Splice Site Alterations and/or Mutation Altering the Codon Tyr1003

Case	Sex	Age, y	Smoking status (pack-years)	Site	Histologic Subtype	*MET* Mutation (Sanger Sequencing)	Detection by NGS	Splicing Alteration Prediction	Comment
1	M	82	Unknown	Lung (surgery)	ADC	c.3028G>A (p.D1010N)	Yes (TS26)	Highly probable	Same mutation found 12 time in patients with lung cancer[5]
2	F	80	Unknown	Lung (biopsy)	ADC	c.3028+3_3028+7del	NA	Highly probable	Substitution at c.3028+3 position found 13 times in patients with lung cancer[5]
3	F	84	Passive smoker	Lymph node (biopsy)	ADC	c.3028+2T>C	Yes (TS26)	Highly probable	Same mutation found 13 times in patients with lung cancer[5]
4	M	78	Former (90)	Bone (biopsy)	ADC	c.2990_3019del (p.S997_T1006del)	No (TS26)	Unlikely but codon Tyr1003 deleted	Similar deletion found 1 time[8]
5	M	69	Former (70)	Lung (biopsy)	NSCLC (NOS)[a]	c.2888-16_2888-1 delinsAAC	Yes (CLV2)	Highly probable	Similar deletion or indel found 8 times in patients with lung cancer[5]

[a]With some sarcomatoid features. Splicing alteration prediction was done with Alamut Visual splicing module software.
MET, mesenchymal epithelial transition factor gene; NGS, next-generation sequencing; M, male; F, female; ADC, adenocarcinoma; NOS, not otherwise specified; NA, not applicable; TS26, TruSight tumor 26; CLV2, Ion Ampliseq Colon and Lung Cancer Research Panel v2.

MET*ex14 Mutation Detection by Targeted NGS*

Two amplicon-based NGS panels were implemented in our laboratory for routine clinical diagnosis of other targetable mutations such as *EGFR* (activating mutations of exons 18–21 and resistance mutation T790M), *KRAS,* and *BRAF* mutations in 2016. We thus considered the possibility of using these panels to also identify *MET*ex14 alterations. Because the five positive *MET*ex14 samples were received before implementation of the NGS, we retrospectively analyzed them with our panels. However, the target region visualization shows that neither panel was able to simultaneously detect all five mutations identified by Sanger sequencing (Fig. 1). In addition, as summarized in Table 1, only three mutations could be correctly detected by NGS. One mutation could not be confirmed on account of insufficient quality of the DNA, and the last one was not detected. Although located in the TS26 target region, the c.2990_3019del NGS false-negative case can be explained by the location of the deletion in the primer binding site of the two adjacent amplicons covering *MET*ex14 (Fig. 2*A* and *B*).

In silico *Comparison of Commercially Available Targeted NGS Panels*

To be more comprehensive, we extended our comparison to six additional commercially available panels dedicated to cancer mutation detection on DNA and based on amplicon technology. The ability of these eight panels to detect *MET*ex14 and other recently described and clinically relevant alteration, such as *ALK, ROS1,* and *EGFR* C797S resistance mutations, was tested *in silico* by using primer location, as described in the Materials and Methods.

Concerning detection of *MET*ex14 alterations, 275 mutated samples (270 extracted from the literature and five identified in this work) corresponding to 151 distinct mutations were listed. These samples were classified into three categories according to the location and/or the size of the alteration: mutations altering the splice acceptor site and/or the splice branch site (AS/Branch site), mutations altering the splice donor site and/or Tyr1003 codon (DS/Y1003) and large deletions (>150 base pairs) (Supplementary Table 1). The ability of the different panels to detect the three classes of *MET*ex14 mutations is summarized in Table 2. Only three panels were able to detect DS/Y1003 mutations, but they were insufficient to detect mutations altering the AS/branch site. Conversely, the three panels detecting the most AS/branch site mutations were unable to detect the DS/Y1003 mutations. In addition, the amplicon-based technology does not allow the detection of large deletions. Finally, none of the panels would have been able to detect more than 63% of the reported cases of *MET*ex14 mutations, and most of them would have detected less than 24% of the expected cases.

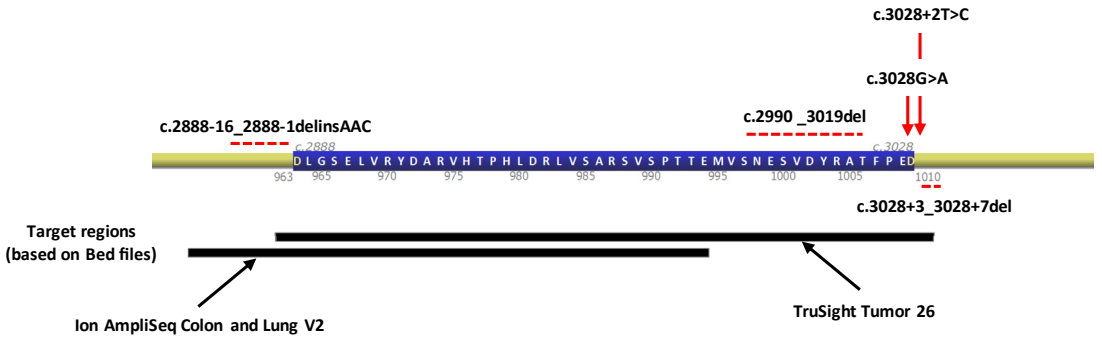

Figure 1. Schematic diagram showing position of indels (*red dashes*) and substitutions (*red arrowheads*) in splice site junctions or codon Tyr1003 of mesenchymal epithelial transition factor gene exon 14 (*MET*ex14) identified in our cohort and target regions of the two panels used in our laboratory.

Concerning the detection of *ALK* mutations involved in TKI resistance, only two panels were able to detect all the major hotspots (Table 3), and only five of them could detect the crucial gatekeeper p.1196 position. It may be noted that one panel was not designed to explore either *MET*ex14 or *ALK* mutations of interest. Among other new druggable alterations, the *EGFR* C797S mutation was virtually detectable with six of eight panels. However,

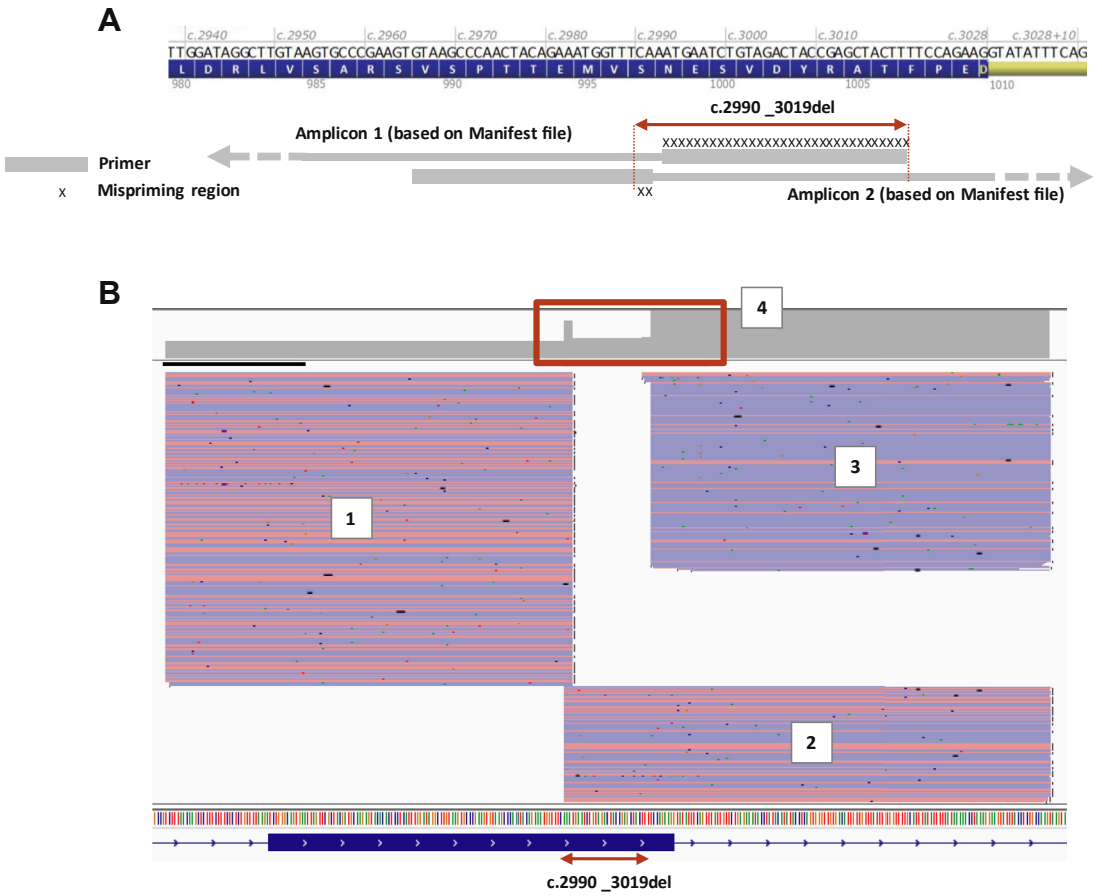

Figure 2. False-negative case with amplicon-based next-generation sequencing technology. (*A*) Juxtaposed view of undetected deletion and primer positions (panel TS26). The deletion was not detected because of its location inside the primer binding site of the two adjacent amplicons covering mesenchymal epithelial transition factor gene exon 14. (*B*) Reads visualization. For the first amplicon, primer hybridization was possible only on wild-type DNA (*1*); amplification of mutated DNA failed, a phenomenon call *allele drop-out*. For the second amplicon, primer hybridization was possible because only the last nucleotides of the primer were located under the deletion. Wild-type (*2*) and mutated (*3*) DNA were amplified, but because of data processing and notably primer trimming, the deletion was removed (*3*) and its presence can only be suspected owing to the low depth of sequencing (*4*).

Table 2. *In Silico* NGS Cancer Panels' Ability to Detect *MET*ex14 Alterations Predicted to Respond to MET Inhibitors

Panel	AS/Branch Site n = 100 (36%)	DS/Y1003 n = 167 (61%)	Large Deletion n = 8 (3%)	Total n = 275 (100%)
TruSight Tumor 26	8 of 100 (8%)	165 of 167 (99%)	0 of 8 (0%)	173 of 275 (63%)
TruSeq Amplicon Cancer Panel	0 of 100 (0%)	167 of 167 (100%)	0 of 8 (0%)	167 of 275 (61%)
Ion AmpliSeq Colon and Lung Panel v2	65 of 100 (65%)	0 of 167 (0%)	0 of 8 (0%)	65 of 275 (24%)
Ion AmpliSeq Cancer Hotspot Panel	65 of 100 (65%)	0 of 167 (0%)	0 of 8 (0%)	65 of 275 (24%)
Tumor Hotspot MASTR Plus	6 of 100 (6%)	167 of 167 (100%)	0 of 8 (0%)	173 of 275 (63%)
ThunderBolts Cancer Panel	57 of 100 (57%)	0 of 167 (0%)	0 of 8 (0%)	57 of 275 (21%)
Accel-Amplicon 56G Oncology Panel v2	8 of 100 (8%)	0 of 167 (0%)	0 of 8 (0%)	8 of 275 (3%)
TruSight Tumor 15	No amplicon targeting *MET*ex14			

NGS, next-generation sequencing; *MET*ex14, mesenchymal epithelial transition factor gene exon 14; MET, mesenchymal epithelial transition factor; TKI, tyrosine kinase inhibitor.

none of these panels was intended to study the *ROS1* gene, so *ROS1* resistance mutations such as G2032R and D2033N could not be detected (data not shown).

Discussion

NGS technology has emerged as a major tool for diagnosis, prognosis, and detection of clinically druggable genetic alterations in cancer specimens. The challenge is to choose adapted technologies to analyze, with confidence, the most useful genomic regions with low-quantity and poor-quality formalin-fixed paraffin-embedded DNA in a short time. These constraints have justified the use of restrained amplicon-based NGS approaches,[8] but they require continuous reevaluation of genomic regions of interest and have now reached limitations justifying taking an interest in other analysis strategies.[7]

This has been illustrated here in particular for the detection of *MET*ex14 mutations. Because of major limitations of the commercial panels we use routinely and the false-negative result, we explored *in silico* the adequacy of other commercially available amplicon-based NGS panels for the detection of these mutations. Similar limitations were observed with all these additional panels and also for other recently described druggable alterations such as *ALK, ROS1,* and *EGFR* C797S resistance mutations. However, these alterations and their clinical relevance were less documented at the time of panel design. Thus, no particular optimization was done to cover them efficiently. Updating an existing panel is time-consuming and expensive; consequently, commercially available NGS panels could be rapidly outdated as new regions to be covered emerge frequently. Now, new panels based on nonamplicon technology or combining DNA and RNA sequencinq are available for the study of formalin-fixed paraffin-embedded samples. They are more adapted to detection of the alteration described here, and especially to the detection of various fusions genes or exons skipping druggable in the NSCLC context. Pending the diagnostic validation and routine use of such panels, complementary technologies should be implemented to minimize loss of information. In our laboratory, we have maintained *MET*ex14 Sanger sequencing concomitantly with NGS analysis, and this recently

Table 3. *In silico* NGS Cancer Panels' Ability to Detect *ALK* Mutations Involved in TKI Resistance

Panel	p.1151 p.1152 p.1156 p.1171	p.1174	p.1180	p.1196 p.1202 p.1203	p.1206 p.1210	p.1269
TruSight Tumor 26	-	X	X	-	-	-
TruSeq Amplicon Cancer Panel	-	X	-	-	-	X
Ion AmpliSeq Colon and Lung Panel v2	X	X	X	X	X	X
Ion AmpliSeq Cancer Hotspot Panel	-	X	X	X	-	-
Tumor Hotspot MASTR Plus	X	X	X	X	X	X
ThunderBolts Cancer Panel	-	X	X	X	X	X
Accel-Amplicon 56G Oncology Panel v2	-	X	X	X	-	X
TruSight Tumor 15	No amplicon targeting *ALK* gene					

Note: X indicates covered position, and - indicates position that is not covered.
NGS, next-generation sequencing; *MET*ex14, mesenchymal epithelial transition factor gene exon 14; *ALK*, anaplastic lymphoma kinase gene; TKI, tyrosine kinase inhibitor.

allowed us to identify a second false-negative case (c.2888-32_2888-9 del) in a patient with NSCLC not otherwise specified with some sarcomatoid features (biopsy sample) that was undetectable with any of the panels discussed in this report.

Here we have highlighted the crucial role of the best knowledge of panels used in routine diagnosis for accurate identification of gene alterations regardless of the technology selected. Information about the exact genomic regions covered and primer positions have to be carefully checked. Frequent reevaluation should be done to remain up-to-date. This is mandatory to deliver the most accurate data to the clinician for therapeutic decisions.

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at http://dx.doi.org/10.1016/j.jtho.2017.07.026.

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