Laboratory Investigation (2021) 101:648–660
https://doi.org/10.1038/s41374-021-00536-2

TECHNICAL REPORT

An improved assay for detection of theranostic gene translocations and *MET* exon 14 skipping in thoracic oncology

Nicolas Piton[1] · Marie-Delphine Lanic[2] · Florent Marguet[1] · Aude Lamy[1] · France Blanchard[1] · Florian Guisier[3] · Mathieu Viennot[2] · Mathieu Salaün[3] · Luc Thiberville[3] · Fabrice Jardin[2] · Jean-Christophe Sabourin[1] · Philippe Ruminy[2]

Received: 20 September 2020 / Revised: 30 December 2020 / Accepted: 30 December 2020 / Published online: 25 January 2021

Abstract

Theranostic translocations may be difficult to detect by routine techniques, especially when specimens are exiguous. We recently demonstrated in a series of translocated lung adenocarcinomas that LD-RT-PCR (*ligation-dependent reverse transcription polymerase chain reaction*) assay could identify *ALK*, *ROS1* and *RET* rearrangements with 64% sensitivity and 100% specificity. Here, we report an upgraded version of this assay used in a routine prospective cohort of lung carcinomas. Newly diagnosed lung carcinomas referred to the Rouen molecular platform between 15/05/2018 and 15/05/2019 for ALK and ROS1 IHC, genotyping (SNaPshot© +/− high-throughput genotyping) and sometimes FISH (standard routine process) were tested prospectively in parallel with the LD-RT-PCR assay designed to detect at one go *ALK*, *ROS1* and *RET* translocations and *MET* exon 14 skipping. 413 tumors from 396 patients were included. LD-RT-PCR had a global sensitivity of 91.43% (standard routine process: 80%), with a specificity of 100%. It detected 15/18 *ALK* and 4/4 *ROS1* translocated tumors, but also 6/6 tumors with *MET* exon 14 skipping retrieved by genotyping. In addition, it retrieved 7 alterations missed by the routine process, then confirmed by other means: 5 *MET* exon 14 skipping and 2 *RET* translocated tumors. Finally, it allowed to deny an effect on *MET* exon 14 skipping for 8 mutations detected by routine genotyping. We successfully implemented LD-RT-PCR in routine analysis. This technique is cheap, fast, sensitive, specific, and easily upgradable (e.g., *NTRK* translocations), but still requires IHC to be performed in parallel. Owing to its advantages, we recommend considering it, in parallel with IHC and genotyping, as an excellent cost-effective alternative, for the systematic testing of lung adenocarcinoma, to FISH and to more expensive and complex assays such as RNA-seq.

Introduction

Lung carcinoma is one of the heaviest cancer burdens worldwide both in terms of incidence and mortality [1, 2]. In the past few years, so-called "genetic theranostic biomarkers" have been extensively described in this disease, especially in adenocarcinoma, mainly involving never or light smokers [3]. These biomarkers are "driver mutations" and can basically be separated in two types: mutations in exonic sequence (mainly involving *EGFR*) or larger DNA modification, comprising translocations (such as *ALK*, *ROS1* and *RET*) and exon skipping, mainly *MET* exon 14 skipping [4–8]. It is important to detect such alterations because they are predictive of good response to targeted therapies, usually better tolerated than conventional chemotherapies [9–12].

Contrary to mutations in exonic DNA, translocations may be difficult to detect by routine techniques such as high-throughput sequencing, immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH), especially when biopsies are exiguous, which is often the case in thoracic oncology and especially in adenocarcinoma, given

✉ Nicolas Piton
nicolas.piton@chu-rouen.fr

[1] Normandie Univ, UNIROUEN, INSERM U1245 and Rouen University Hospital, Department of Pathology, F76000, Normandy Centre for Genomic and Personalized Medicine, Rouen, France, F-76000 Rouen, France

[2] INSERM U1245, Cancer Center Henri Becquerel, Institute of Research and Innovation in Biomedicine (IRIB), University of Normandy, UNIVROUEN, Rouen, France

[3] Normandie Univ, UNIROUEN, EA4108 LITIS lab, CHU Rouen, Department of Pneumology and Inserm CIC-CRB 1404, F-76000 Rouen, France

their peripheral localization. In many centers, ALK and ROS1 IHC are performed when a diagnosis of "non-squamous non-small cell" lung carcinoma is made, and if negative, it is inferred that *ALK* and *ROS1* are not rearranged, except if the patient is a non-smoker or if signet ring morphology is observed [13]. In case of clearly positive ALK IHC, a validation method (FISH or RNA-based analysis) is not usually performed, but it is required for ROS1 as IHC signal is not completely specific for *ROS1* translocation.

Distinctions have been made between variants of *ALK* translocations regarding the aggressivity of the disease and the efficacy of targeted therapy [14, 15]. However, usual techniques such as IHC and FISH are unable to stratify patients according to the precise breakpoint and therefore make it impossible to personalize the therapeutic approach and to predict tumor behavior. Conversely, as there is no recommended immunostaining assay for *RET*, this translocation will not be detected, unless a test, FISH or RNA-based approach, is specifically asked [16–19].

Finally, *MET* exon 14 skipping is also challenging to diagnose at the DNA level, mainly because the underpinning molecular alterations are multiple, involving indels and base substitutions at both the splice acceptor and donor sites of exon 14 (Fig. 1). The difficulty usually resides in the fact that intronic sequences are less commonly analyzed and that bioinformatics is sometimes incapable to predict confidently the functional consequences of the alteration on gene skipping. Conversely, some of the *MET* alterations detected at the DNA level result in incomplete skipping of around 80% [20], indicating therefore that analyses at both the RNA and DNA levels are sometimes required not to miss any case [8].

FISH is a time-consuming and expensive technique which is sometimes difficult to interpret. In addition, its validity depends on pre-analytic conditions. Finally, specificity is unperfect and the break-apart probes approach is unable to identify the partner gene, contrary to fusion probes, but the latter are impractical routinely because of the multiplicity of potential partner genes. High-throughput RNA-based techniques are also expensive, require expertise in bioinformatics and usually generate "over information".

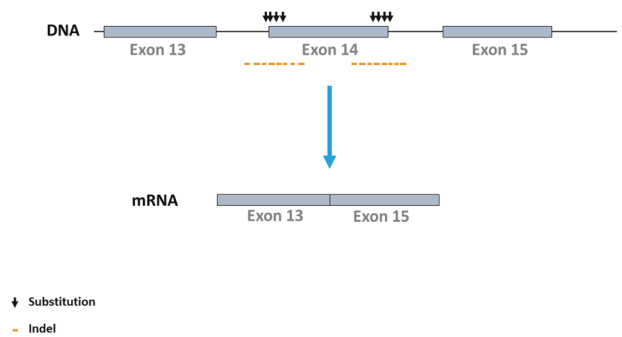

Fig. 1 Location of *MET* gene alterations inducing exon 14 skipping. *MET* exon 14 skipping is caused by mutations at splice sites flanking this exon. It consists of point mutations or indels which can be located in introns, quite far from exon 14 and therefore difficult to detect. The advantage of assays using mRNA rather than DNA is that they detect directly the consequence of the alteration, and therefore can ascertain its biological impact without having to predict the effect according to previous reports or in silico. This figure is adapted from [34] and [50].

We recently demonstrated in a small retrospective series of translocated lung adenocarcinomas of known status (14 *ALK*, 14 *ROS1* and 1 *RET* translocations) that a cheap 47-probe LD-RT-PCR (*ligation-dependent reverse transcription polymerase chain reaction*) assay could identify *ALK*, *ROS1* and *RET* rearrangements on formalin-fixed paraffin-embedded (FFPE) tissue with a sensitivity of 64% and a 100% specificity and with availability of results in 2 days on average, regardless of RNA extraction procedure [21]. This assay has been developed in the INSERM U1245 research group and is successfully used daily to classify some hematologic neoplasms [22, 23].

Here, we report an upgraded and updated version of this assay used in a routine prospective cohort of 413 newly diagnosed lung adenocarcinomas. Compared to our previous publication, 43 potential partners have been added to the reaction mixture, including *MET* translocation partners [24, 25], following the evolution of the scientific literature. In addition, we sought to determine whether this technique could easily detect *MET* exon 14 skipping and therefore we modified the assay for this purpose.

Materials and methods

Ethics

Ethical approval was obtained according to the agreement of the tumor biobank of Rouen University Hospital (tissue sample collection no DC2008-689) by the institutional review board of Rouen University Hospital and the French Ministry of Scientific Research.

Design of the study

Prospective cases of newly diagnosed lung carcinomas referred for molecular testing to the French National Cancer Institute molecular platform of Rouen University Hospital between 15/05/2018 and 15/05/2019 were in parallel tested with the LD-RT-PCR assay (Fig. 2). For ALK and ROS1, IHC was performed first as routinely, and FISH was then ordered to detect gene rearrangement whenever the immunoassay was positive.

First, IHC slides for ALK, ROS1 and PDL1 were made from FFPE tumor. Then, serial unstained sections for both genotyping and LD-RT-PCR were prepared in the same

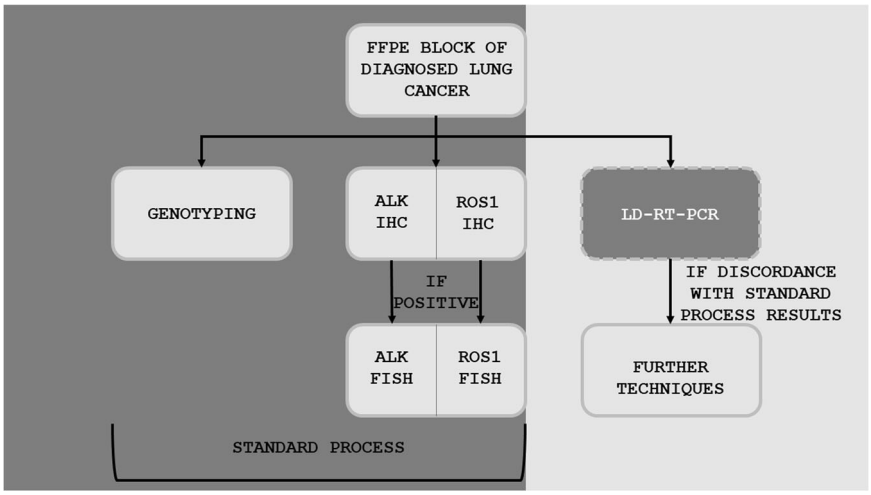

Fig. 2 Design of the study. New lung cancers eligible for genotyping (non-small cell non-squamous lung cancer or occurring in a never or light smoker) referred to the molecular platform were tested in parallel by the routine process (i.e., genotyping by high-throughput analysis or SNaPshot multiplex kit, ALK and ROS1 IHC) and the LD-RT-PCR assay. Whenever ALK or ROS1 IHC was positive, a break-apart FISH was performed. If results of the routine process and those of LD-RT-PCR were discordant, further molecular techniques were performed. FFPE formalin-fixed paraffin-embedded, IHC immunohistochemistry. LD-RT-PCR ligation-dependent reverse transcription polymerase chain reaction.

time to avoid tissue loss when leveling the paraffin block. In brief, serial sections of FFPE tumors were cut from a paraffin block representative of lesion and placed on glass slides: a 4-mm-thick section was stained with hematoxylin & eosin (H&E) for microscopic examination. The following sections were processed for preparation of tumor nucleic acids. The microtome razor blade was changed between each FFPE tumor sample, and the paraffin sections were processed individually to avoid cross contamination. H&E preparation enabled tumor area delimitation and visual estimation of tumor cell percentage simultaneously. The tumor area was macro-dissected on a 10-µm-thick section placed on a glass slide using a single-use sterilized scalpel. Both DNA (for classic genotyping) and RNA (for LD-RT-PCR) were extracted from the 10-µm-thick section with the Maxwell 16 LEV RNA FFPE kit (Promega©, Madison, Wisconsin).

For discordant cases between the classic routine sequential analysis and the LD-RT-PCR, we aimed at characterizing precisely the molecular alterations using different techniques. For detection of *RET* translocation, break-apart FISH probes were used (Caen University Hospital, France) as no validated and reliable IHC antibody has been commercialized. For cases with suspected *MET* exon 14 skipping, we sent the samples to Lille University Hospital, France (details below), as our panel does not fully sequence *MET* introns. Finally, for 3 cases, we performed in-house 5'RACE PCR (rapid amplification of cDNA-ends by polymerase chain reaction, for research use only).

IHC assays

For detection of ALK protein, we used the Ventana ALK (D5F3) CDx Assay (Roche©, Bâle, Switzerland), whereas ROS1 protein was detected using the ROS1 D4D6 Rabbit monoclonal antibody #3287 (Cell Signaling Technology©, Danvers, Massachusetts), following the routine procedure for all newly diagnosed lung adenocarcinoma.

For ALK and ROS1 detection, positivity was defined as a cytoplasmic signal, regardless of the intensity of the staining or the percentage of the stained tumor cells (test considered either positive or negative). As stated above, we did not perform immunostaining for RET protein detection.

FISH

We performed FISH using break-apart probes to detect *ALK* and *ROS1* translocations: using Vysis ALKBreak Apart FISH Probe Kit for ALK and ZytoLight SPEC ROS1 Dual Color Break Apart Probe kit (CliniSciences©, Nanterre, France) for ROS1. FISH for *RET* was performed using the ZytoLight SPEC RET Dual Color Break Apart Probe kit (CliniSciences©, Nanterre, France). Cells were considered as rearranged when at least one set of orange and green signals were two or more signal diameters apart, or when there was a single orange signal without a corresponding green signal in addition to fused and/or broken apart signals, according to the manufacturer's instructions. At least 100 tumor nuclei were analyzed, and a case was considered

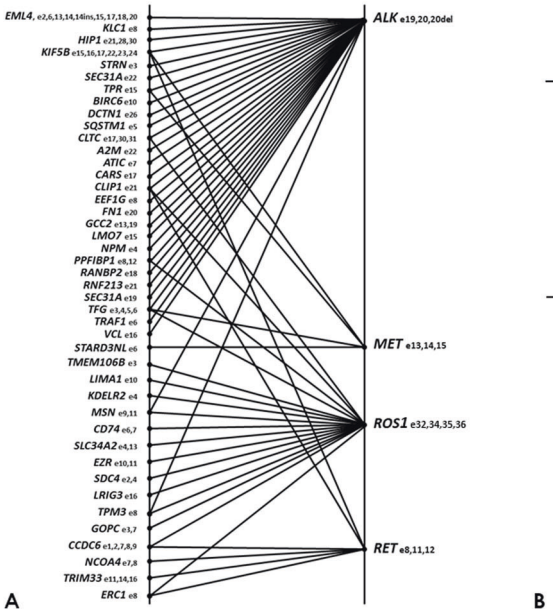

Fig. 3 Design of the LD-RT-PCR assay. A Schematic representation of the assay for the detection of fusion transcripts of *ALK*, *ROS1*, *RET* and *MET*. LD-RT-PCR probes were designed for 91 different genes to target 87 rearrangements. For most genes, multiple probes were designed on different exons to target different transcripts that result from the distribution of the genomic breakpoints within different introns. **B** Principle of the detection of *MET* exon 14 skipping by the LD-RT-PCR assay. Specific probes of the 3' part of *MET* exon 13 and 14 and of the 5' part of *MET* exon 14 and 15, with various lengths. When no *MET* exon skipping was present, two different PCR products of 52 + 49 base pairs and 55 + 53 base pairs were observed. When a MET allele harboring exon 14 skipping was present, a third PCR product with a length of 52 + 53 base pairs was observed. bp base pairs, F fluorophore, LD-RT-PCR ligation-dependent reverse transcription polymerase chain reaction.

as positive when the number of rearranged neoplastic nuclei was at least 15% of observed neoplastic nuclei.

Genotyping

For cases diagnosed at Rouen University Hospital containing more than 5% tumor cells, high-throughput sequencing was performed (theoretical lower limit of detection estimated at 2.5% variant allele frequency). This assay was performed from FFPE tumor tissue, using the Tumor Hotspot MASTR Plus (Agilent©, Santa Clara, California) on a MiSeq sequencing machine (Illumina©, San Diego, California). The following gene regions were sequenced: *AKT1* (exon 3), *ALK* (exons 20–29), *BRAF* (exons 11,15), *CDKN2A* (exons 1–3), *CTNNB1* (exon 3), *DDR2* (exons 3–18), *EGFR* (exons 18–21), *ERBB2* (exons 19–21), *ERBB4* (exons 10–12), *FGFR2* (exons 7, 10, 12), *FGFR3* (exons 7, 9, 14, 15), *H3F3A* (exon 2), *HIST1H3B* (exon 1), *HRAS* (exons 2–4), *IDH1* (exon 4), *IDH2* (exon 4), *KIT* (exons 8, 9, 10, 11, 13, 14, 17, 18), *KRAS* (exons 2–4), *MAP2K1* (exons 2, 3), *MET* (exons 2, 10, 14–20), *NRAS* (exons 2–4), *PDGFRA* (exons 12, 14, 18), *PIK3CA* (exons 2, 3, 10, 11, 21), *PIK3KR1* (exons 11, 12, 13), *PTEN* (exons 1–9) and *STK11* (exons 1–9). Results were analyzed with BWA-GATK©, VarScan2© and Alamut HT© bioinformatic software.

According to the policy of the molecular platform, for cases referred for genotyping from outside Rouen University Hospital or affiliated hospitals, or cases containing less than 5% tumor cells, only hotspot mutation analysis was performed by SNaPshot multiplex kit (ThermoFisher Scientific©, Waltham, Massachusetts), enabling only detection of mutations in the following codons: *EGFR* c.2155G, *EGFR* c.2156G, *EGFR* c.2369C, *EGFR* c.2573T, *EGFR* c.2582T, *KRAS* c.34G, *KRAS* c.35G, *KRAS* c.37G, *KRAS* c.38G, *BRAF* c.1799T, deletion or substitution of *EGFR* exons 19 and 20 and *ERBB2/HER2* exon 20.

Finally, discordant cases were analyzed by high-throughput genotyping at Lille University Hospital with an in-house gene panel using multiplex PCR Ampliseq with Ion S5 XL system (Thermo Fischer Scientific©, Waltham, Massachusetts). Data were analyzed using Torrent Suite V5.2 software (Thermo Fischer Scientific©, Waltham, Massachusetts) and DVD.

LD-RT-PCR assay

The principle of the LD-RT-PCR assay and details regarding tissue processing are available in our previous paper [21]. The improvements to the assay (A: increase in the number of probes in the mix and B: detection of *MET* exon 14 skipping) are illustrated in Fig. 3.

Table 1 Main clinical and histological data of patients and tumors included in the study.

Mean age (in years) at molecular testing	65 (SD = 11)
	Number of cases (%)
Sex	
Male	256 (65%)
Female	140 (35%)
Tumor site	
Lung	270 (65,4%)
Metastasis	141 (34.1%)
Pleura	49
Lymph node	45
Central nervous system	17
Bone	17
Skin	6
Liver	3
Pericardium	2
Adrenal gland	1
Muscle	1
Missing data	2 (0.5%)
Tumor sample	
Resection specimen	143 (34.6 %)
Bronchial biopsy	113 (27.4 %)
Surgical biopsy	94 (22.8%)
Transparietal biopsy	28 (6.8%)
Pleural effusion	15 (3.6%)
Lymph node aspiration	14 (3.4%)
Pericardial effusion	2 (0.5%)
Bronchial brush	1 (0.2%)
Bronchial aspiration	1 (0.2%)
Missing data	2 (0.5%)
Histological type of the tumor	
Adenocarcinoma	390 (94.4%)
Undifferentiated carcinoma	6 (1.5%)
Squamous cell carcinoma	5 (1.2%)
Large cell neuroendocrine carcinoma	4 (1%)
Sarcomatoid carcinoma	3 (0.7%)
Carcinoma “not otherwise specified”	1 (0.2%)
Neuroendocrine small cell carcinoma	1 (0.2%)
Adenoid cystic carcinoma	1 (0.2%)
Pulmonary blastoma	1 (0.2%)
Mucoepidermoid carcinoma	1 (0.2%)
Tumor cell content	
0–5%	15 (3.6%)
5–15%	31 (7.5%)
15–25%	140 (33.9%)
25–50%	114 (27.6%)
50–100%	112 (27.1%)
Missing data	1 (0.2%)

Reverse transcription was performed with the Superscript VILO cDNA Synthesis kit (ThermoFischer Scientific©, Waltham, Massachusetts). For this work, samples were batched up to 40 to save on labor and costs, but it was also possible to test a single sample when needed. cDNA were next incubated 1 h at 60 °C with a mix of ligation-dependent PCR oligonucleotide probes, including universal adapter sequences and random sequences of 7 nucleotides as unique molecular identifiers (UMI) in 1x SALSA MLPA buffer (MRC Holland©, Amsterdam, the Netherlands), ligated using the thermostable SALSA DNA ligase kit (MRC Holland©, Amsterdam, the Netherlands), and amplified by PCR using barcoded primers containing P5 and P7 adapter sequences with the Q5 hotstart high fidelity master mix (NEB©, Ipswich, Massachusetts). Amplification products were next purified using AMPure XP beads (Beckman Coulter©, Brea, California) and analyzed using a MiSeq sequencer (Illumina©, San Diego, California). Sequencing reads were de-multiplexed using the index sequences introduced during PCR amplification, aligned with the sequences of the probes and counted. All results were normalized according to the UMI sequences to avoid PCR amplification bias. Sequencing results were blindly interpreted. For *MET* exon 14 skipping detection, specific LD-RT-PCR probes of the 3’ part of *MET* exon 13 and 14 and of the 5’ part of *MET* exon 14 and 15, with various lengths were designed. When no *MET* exon skipping was present, two different PCR products of $52 + 49$ base pairs and $55 + 53$ base pairs were observed. When a MET allele harboring exon 14 skipping was present, a third PCR product with a length of $52 + 53$ base pairs was expected.

LD-RT-PCR results were analyzed using a home-made dedicated bioinformatic pipeline. Briefly, sequences from the different samples were first demultiplexed from the fastQ files using the molecular barcodes of the 8 base pairs introduced at the PCR amplification step. Sequences were next aligned with the gene specific part of the LD-RT-PCR probes to characterize the translocation partners. For each junction, an estimation of the actual number or ligations was provided by the quantification of the random unique molecular index (UMI) present within each 5’ LD-RTPCR probe, after correction of sequencing errors. For each case, the pipeline thus returned the total number of reads, a list of junctions, the number of times that each junction was sequenced, and the estimated number of cDNA molecules which were detected, evaluated using the frequencies of the UMI sequences.

To validate the LD-RT-PCR assay, controls (one positive (EML4-ALK) and one negative RNA) were systematically tested together with routine samples during the whole course of the study. For each sample, the integrity of the RNA samples was validated through the obtention of

positive signals corresponding to the normal MET mRNA junctions (exon13–14 and exon14–15).

Assessment of diagnostic tools

Performance characteristics (sensitivities, specificities, positive predictive values, negative predictive values and accuracies) as well as disease prevalences and their 95% confidence intervals were calculated using the free online tool MedCalc Statistical Software version 16.4.3 (MedCalc Software bv, Ostend, Belgium; https://www.medcalc.org; 2016).

Results

Clinical and histological characteristics

From 15/05/2018 to 15/05/2019, 413 lung carcinomas from 396 patients were referred for genotyping and therefore included in our study. Clinical and histological data are summarized in Table 1. Mean age at genotyping was 65 years (standard deviation = 11). 65% of patients ($n = 256$) were male and 35% ($n = 140$) were female. Primary site was tested in 65.4% ($n = 270$) while metastasis in 34.1% ($n = 141$), with missing data for 2 cases. Tumor tissue was obtained by surgery in 57.4% (resection specimen or surgical biopsy), bronchial or transparietal biopsies in 34.1% and cytology samples in only 8%. Regarding the histopathological nature of the tumors according to the 2015 World Health Organization classification [26], the overwhelming majority were adenocarcinomas (94.4%). Most tissue samples (88.8%) contained more than 15% of tumor cells, with only a few cases (3.6%) with less than 5% tumor cells.

Molecular characteristics

310 cases (75.06%) were genotyped by high-throughput sequencing while 103 (24.93%) by SNaPshot multiplex kit (ThermoFisher Scientific©, Waltham, Massachusetts) only. All were tested by ALK and ROS1 IHC and by LD-RT-PCR. Following discordances between the different assays, 2 cases were tested by *RET* FISH, 3 by 5'RACE for *ALK*, 2 by Lille high-throughput sequencing and 1 case first tested only by SNaPshot multiplex kit (ThermoFisher Scientific©, Waltham, Massachusetts), was then genotyped by Rouen high-throughput sequencing.

Molecular alterations observed are summarized in Table 2. A *KRAS* mutation was the most frequent alteration retrieved (35.99%), and *EGFR* mutation was present in 11.59%. Eighteen tumors harbored an *ALK* translocation (4.35%; 95% Confidence Interval (CI) for disease prevalence = [2.60%–6.80%]), 4 harbored a *ROS1* translocation (0.97%; 95% CI = [0.26%–2.46%]) and 2 harbored a *RET* translocation (0.48%; 95% CI = [0.06%–1.74%]). Nineteen cases harbored a *MET* mutation (4.60%), with only 11 causing exon 14 skipping (2.66%; 95% CI = [1.34%–4.72%]). No tumor was detected with two or more known "driver mutations".

Table 2 Mutational status of tumors included in the study.

Mutational status	Number of cases (%)
KRAS mutation	149 (35.99%)
No mutation retrieved	108 (26.09%)
EGFR mutation	48 (11.59%)
Other mutation	39 (9.42%)
MET mutation	19 (4.60%)
MET exon 14 skipping mutation	11 (2.7%)
MET variant of unknown significance alone	8 (1.94%)
BRAF mutation	19 (4.60%)
ALK translocation	18 (4.35%)
ERBB2 mutation	5 (1.21%)
Technical failure	6 (1.45%)
ROS1 translocation	4 (0.97%)
RET translocation	2 (0.48%)

Data are compiled from the results of IHC, FISH, genotyping and LD-RT-PCR. For the sake of clarity, double mutations have been omitted, explicating the reason why the sum is over 413 (1 case with a *KRAS* mutation and a *MET* variant of unknown significance; 1 case with a *KRAS* mutation and a *BRAF* mutation; 1 case with an *ALK* translocation and a *MET* variant of unknown significance).

ALK, ROS1 and RET translocations

The routine process for detecting translocations, i.e., IHC and FISH, retrieved 18 *ALK* and 4 *ROS1* translocations (Fig. 4A). As *RET* or *MET* translocations were not systematically looked for, no such alteration was observed. In contrast, LD-RT-PCR retrieved 15/18 *ALK* and 4/4 *ROS1* translocations and detected 2 *RET* translocations (Table 3). No *MET* translocation was observed.

ALK translocated cases missed by LD-RT-PCR (Table 3, cases ALK-B, ALK-Q and ALK-R) were all positive for both IHC and FISH. They were subsequently confirmed by an in-house 5'RACE PCR identifying two complex *ALK* translocations (EML4 exon 7 – ALK exon 20 with deletion of 30 base pairs; EML4 exon 13 - ALK exon 20 with 18 base pairs in ALK intron 19–20) and a partner gene never described in literature (TRIM24 exon 12 – ALK exon 20). *RET* translocated cases detected by LD-RT-PCR were subsequently confirmed by a *RET* break-apart FISH assay performed at Caen University Hospital.

Regarding *ALK*, *ROS1* and *RET* translocations only, LD-RT-PCR had the following diagnosis performance: sensitivity = 87.5% (95% CI = [67.64%–97.34%]), specificity = 100% (95% CI = [99.06%–100.00%]), positive predictive value = 100%, negative predictive value = 99.23 % (95%

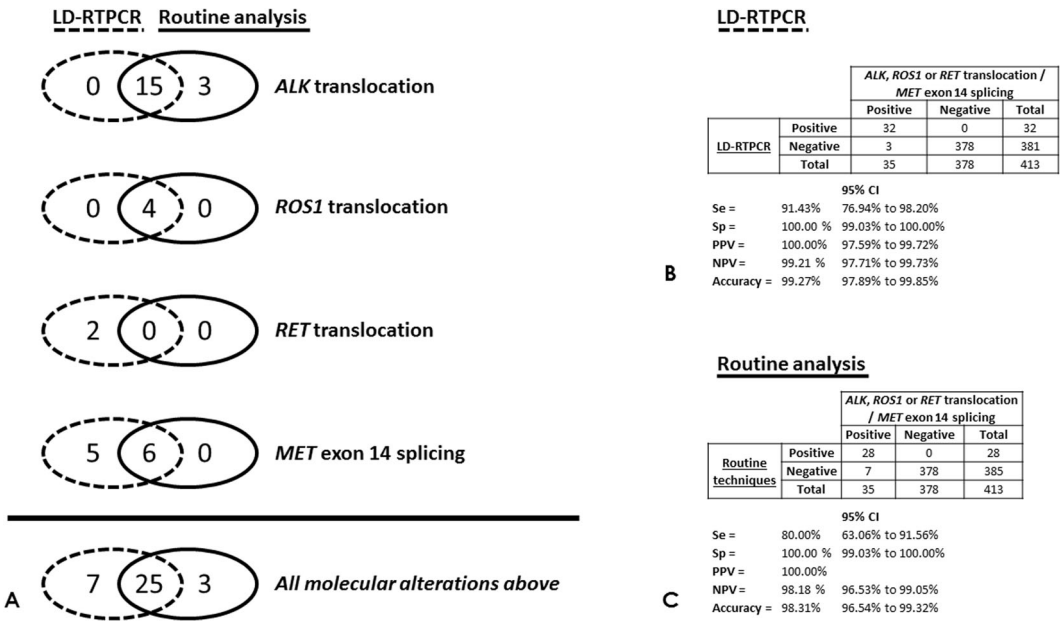

Fig. 4 Summary of the results. A Comparative evaluation of the positive cases retrieved by LD-RT-PCR, by routine analysis and by both for *ALK*, *ROS1*, *RET* translocation, *MET* exon 14 skipping and in total. **B** Contingency table used to calculate the performance characteristics of LD-RT-PCR to detect *ALK*, *ROS1*, *RET* translocation and *MET* exon 14 skipping. **C** Contingency table used to calculate the performance characteristics of routine analysis to detect *ALK*, *ROS1*, *RET* translocation and *MET* exon 14 skipping. Se sensitivity, Sp specificity, PPV positive predictive value, NPV negative predictive value, 95% CI 95% confidence interval, LD-RT-PCR ligation-dependent reverse transcription polymerase chain reaction.

CI = [97.83%–99.73%]) and accuracy = 99.27% (95% CI = [97.89%–99.85%]).

MET exon 14 skipping

Results for MET are summarized in Table 4. Overall, 19 cases harbored a *MET* mutation. Fourteen tumors harboring *MET* mutations were identified by the routine process (cases MET-A to MET-E and MET-K to MET-S). Among these cases, LD-RT-PCR diagnosed 6 (cases A to E and case K) harboring a *MET* mutation leading to exon 14 skipping (Fig. 4A). Furthermore, it retrieved 5 cases without any *MET* mutation observed on genotyping assays but containing *MET* exon 14 skipping (cases MET-F to MET-J). One of these cases had only been tested by SNaPshot multiplex kit (ThermoFisher Scientific©, Waltham, Massachusetts), according to the laboratory procedures regarding external samples, and a *MET* mutation was retrieved in retrospect using Rouen high-throughput genotyping (case MET-J). For the remaining 4 cases on which high-throughput genotyping was negative, tumor DNA was sent to Lille University Hospital and for each case, a *MET* mutation was retrieved (cases MET-F to MET-I). These mutations were interpreted as "likely to lead to exon 14 skipping". Cases MET-L to MET-S were negative for LD-RT-PCR: case MET-L failed LD-RT-PCR but its effect was interpreted very differently according to the prediction tool used dbSNP [27], ClinVar [28], COSMIC [29] and cBioPortal [30, 31], "deleterious" according to SIFT [32] and "disease causing" according to Mutation Taster [33]. According to Alamut Visual© version 2.10 (Interactive Biosoftware, Sophia Genetics, Lausanne, Switzerland), this mutation could lead to exon 14 skipping. Regarding cases MET-M to MET-S, the location of the *MET* mutation was unlikely to lead to exon 14 skipping.

Comparison between overall performance characteristics of LD-RT-PCR and routine process

Performance characteristics of LD-RT-PCR when combining detection of *ALK*, *ROS1*, *RET* and *MET* translocation and *MET* exon 14 skipping are compiled in Fig. 4B. Sensitivity of LD-RT-PCR was 91.43% (95% CI = [76.94%–98.20%]) while that of routine process was 80% (95% CI = [63.06%–91.56%]). Both processes were 100% specific, meaning that they did not retrieve any molecular alterations which were not present in the samples.

Finally, 20 cases (4.84%) failed analysis with LD-RT-PCR.

Response to targeted therapy

Ten patients with *ALK* ($n = 8$) *or ROS1* ($n = 2$) translocated tumors had stage IV disease and received targeted therapy with crizotinib ($n = 7$) or alectinib ($n = 3$) as first-line treatment. The response rate was 100%; Kaplan–Meier

Table 3 Clinical, histological and molecular data of the *ALK*, *ROS1* or *RET* translocated cases.

Case	Sex	Age	Sample	Location	Histology	Tumor cells %	Routine IHC / FISH	LD-RT-PCR	5′ transcript	3′ transcript
ALK-A	M	86	RS	L	ADC	50–100	+/+	+	EML4 exon 2	ALK exon 20
ALK-B	M	65	RS	L	ADC	25–50	+/+	−	EML4 exon 13	ALK exon 20
ALK-C	F	46	BB	L	ADC	15–25	+/+	+	EML4 exon 13	ALK exon 20
ALK-D	M	72	BB	L	ADC	5–15	+/+	+	EML4 exon 13	ALK exon 20
ALK-E	F	54	RS	L	ADC	25–50	+/+	+	EML4 exon 13	ALK exon 20
ALK-F	M	61	BB	L	ADC	25–50	+/+	+	EML4 exon 13	ALK exon 20
ALK-G	F	86	BB	L	ADC	15–25	+/+	+	EML4 exon 6	ALK exon 20
ALK-H	F	28	SR	P	ADC	15–25	+/+	+	EML4 exon 13	ALK exon 20
ALK-I	M	77	BB	L	ADC	15–25	+/+	+	EML4 exon 17	ALK exon 20
ALK-J	M	66	BB	L	ADC	15–25	+/+	+	EML4 exon 6	ALK exon 20
ALK-K	F	83	RS	L	ADC	50–100	+/+	+	EML4 exon 13	ALK exon 20
ALK-L	M	75	RS	L	ADC	0–5	+/+	+	EML4 exon 6	ALK exon 20
ALK-M	M	71	BB	L	ADC	15–25	+/+	+	HIP1 exon 21	ALK exon 20
ALK-N	M	68	RS	L	ADC	50–100	+/+	+	EML4 exon 18	ALK exon 20
ALK-O	F	44	SR	LN	ADC	25–50	+/+	+	EML4 exon 13	ALK exon 20
ALK-P	F	44	RS	L	ADC	25–50	+/+	+	EML4 exon 13	ALK exon 20
ALK-Q	M	64	SR	P	ADC	15–25	+/+	−	EML4 exon 7	ALK exon 20
ALK-R	F	68	RS	L	ADC	25–50	+/+	−	TRIM24 exon 12	ALK exon 20
ROS1-A	F	85	RS	L	ADC	50–100	+/+	+	EZR1 exon 10	ROS1 exon 32
ROS1-B	F	52	SR	P	ADC	50–100	+/+	+	CD74 exon 6	ROS1 exon 34
ROS1-C	M	97	SR	P	ADC	0–5	+/+	+	CD74 exon 6	ROS1 exon 32
ROS1-D	F	60	SR	P	ADC	15–25	+/+	+	SDC4 exon 2	ROS1 exon 32
RET-A	F	38	SR	LN	ADC	15–25	NA	+	KIF5B exon 15	RET exon 12
RET-B	M	65	BB	L	ADC	15–25	NA	+	CCDC6 exon 1	RET exon 12

Cases ALK-O and ALK-P are from the same patient. Age is indicated in years.

M male, *F* female, *RS* resection specimen, *BB* bronchial biopsy, *SB* surgical biopsy, *L* lung, *P* pleura, *LN* lymph node, *ADC* adenocarcinoma, + positive, − negative, *NA* not applicable.

estimates for progression free survival and overall survival were 11.1 months (95% CI 9.0 - Not Reached) and 31.2 months (95% CI 23.2 - Not Reached), respectively. Two patients received lorlatinib as second-line treatment. Both had partial response as best tumor response, with 8.0 and 7.1 months progression free survival, respectively.

One patient with *RET* translocated tumor received selpercatinib, with partial response still ongoing after a 20-month follow-up.

One patient with *MET* exon 14 skipping tumor (case MET-L) received crizotinib after failure of chemotherapy and immunotherapy, with progressive disease as best tumor response.

For the remaining patients, clinical data were not available.

Discussion

This work enabled us to test on a large scale and in one-year routine conditions of thoracic oncology an innovative technique specifically designed to detect at one go *ALK*, *ROS1* and *RET* translocations and *MET* exon 14 skipping. The LD-RT-PCR assay has been modified and improved compared to our last publication [21]: 43 potential partners have been added to the reaction mixture (Fig. 3A) and the assay was also designed to detect *MET* exon 14 skipping. LD-RT-PCR appears to be a powerful diagnostic tool as well as being fast and cheap, but it missed 3 ALK translocated cases which were IHC positive, and therefore cannot replace IHC. It enables identification of the partner gene involved in translocation, which can be useful to adapt patients' treatment and monitoring [14, 15]. Finally, this test is close to being a functional one because it provides information more on the potential consequence of a *MET* mutation rather than on the mutation itself. Therefore, it appears more helpful than in silico prediction - for example SIFT [32] or MutationTaster [33] - or literature databases such as dbSNP [27], ClinVar [28], COSMIC [29] or cBioPortal [31] to assess easily whether the tumor should be considered as *MET* mutated, and therefore eligible for targeted therapy.

Table 4 Clinical, histological and molecular data of the *MET* cases (either leading to exon 14 skipping or not).

Case	Sex	Age	Sample	Location	Histology	Tumor cells %	MET mutation	Routine process	LD-RT-PCR (exon 14 skipping only)
MET-A	F	67	BB	L	ADC	15–25	c.3082+1G>A	+	+
MET -B	F	71	RS	L	ADC	25–50	c.2942-2A>C	+	+
MET -C	M	68	C	LN	ADC	15–25	c.3082+1G>A	+	+
MET -D	M	78	RS	L	ADC	15–25	c.2942-3_2962del	+	+
MET -E	F	63	SB	P	ADC	15–25	c.3082G>C	+	+
MET -F	M	69	RS	L	ADC	50–100	c.2942-36_2942-14del	−	+
MET -G	M	70	RS	L	ADC	15–25	c.2942-36_2942-14del	−	+
MET -H	F	63	RS	L	ADC	15 to 25	c.2942-6_2942-2delinG	−	+
MET -I	F	63	RS	L	ADC	15 to 25	c.2942-6_2942-2delinG	−	+
MET -J	M	73	RS	L	ADC	50 to 100	c.3082+2T>C	−	+
MET -K	M	73	RS	L	ADC	15 to 25	c.3082+1G>A and c.252C>G	+	+
MET -L	F	72	BR	L	ADC	15 to 25	c.3061T>C	+	−
MET -M	M	67	BR	L	ADC	50 to 100	c.3308_3311delinsAAAA	+	−
MET -N	M	47	BR	L	ADC	15 to 25	c.25C>T	+	−
MET -O	F	84	BR	L	ADC	5 to 15	c.3890G>A	+	−
MET -P	M	71	BR	L	ADC	15 to 25	c.128T>C	+	−
MET -Q	M	74	BR	L	ADC	15 to 25	c.764G>T	+	−
MET -R	M	73	SB	L	ADC	15 to 25	c.467C>T	+	−
MET -S	F	76	RS	LN	ADC	15 to 25	c.974C>T	+	−

Cases MET-F and MET-G are from the same patient (the difference in age is due to a different sample time), as are cases MET-H and MET-I. Sample MET-K presented with two different *MET* mutations. Age is indicated in years. We report here all the *MET* mutations retrieved in our series, even those who are known not to lead to exon 14 skipping, which are useful to assess the specificity of LD-RT-PCR (no false positive result).

M male, *F* female, *RS* resection specimen, *BB* bronchial biopsy, *C* cytology, *SB* surgical biopsy, *L* lung, *P* pleura, *LN* lymph node, *ADC* adenocarcinoma, + positive, − negative, *NA* not applicable.

These prospective results on the detection of translocations are more robust than those published in 2018 in our retrospective series of positive cases. The sensitivity for detecting *ALK*, *ROS1* and *RET* translocations (excluding *MET* exon 14 skipping, not included in our first assay) was 87.50%, compared to 64% previously [21]. Not surprisingly, the specificity was unchanged at 100%, and the fact that we did not retrieve any gene translocation in tumors harboring any other driver mutation (e.g., *KRAS* or *EGFR* mutations) strengthens the validity of the specificity calculation. Many different factors can explain this significant improvement in our results regarding sensitivity. The main point is that in our retrospective series, especially for exiguous tissue samples, exhaustion of tumor tissue was maximal because of re-cut of the paraffin block and RNA were more degraded since some blocks were made more than 2 years before analysis. In contrast, our prospective protocol enabled maximal RNA quality and minimal loss of tissue since slides were cut in the same time as those intended for genotyping. Finally, one cannot exclude the fact that our retrospective series contained some false positive cases with limited positivity in IHC and FISH, therefore artificially decreasing the sensitivity of our assay because some cases did not harbor any gene translocation. In contrast, the prospective series reported above was certainly more robust as improvements have been made, especially in ALK and ROS1 immunoassays, to obtain clear positivity of translocated tumor cells. It is noteworthy that the improvements in the LD-RT-PCR assay between our previous article and this one (i.e., addition of 43 potential gene partners) did not allow the detection of additional positive cases (except for *MET* mutations), as all the gene partners retrieved (Table 3) would have been detected by the first assay [21].

One *ALK* translocation missed by LD-RT-PCR involved the partner *TRIM24* exon 12, which has never been described in the literature (case ALK-R). Following this observation, we were able to add a probe covering this partner to the mix. For the 2 remaining missed cases (ALK-B and ALK-Q), the translocation was complex, explaining the fact that LD-RT-PCR could not detect them. Unfortunately, we were unable to ensure the effect of targeted therapy on these mutations because both cancers were resected at early stage and no adjuvant therapy was prescribed. These complex translocations are likely to be so rare, unique (as we did not retrieve them in the literature and

molecular databases) and complex that such cases will probably never be detected by our assay, fully justifying the recommendation for maintaining the IHC assays as the detection of the chimeric proteins is unlikely to be affected by the breakpoint. The main limit of LD-RT-PCR is that two probes, one on each partner gene, are necessary to detect a rearrangement. Thus, it cannot detect atypical translocations if they have not been anticipated during the design of the assay. It also requires a minimal amount of nucleic acids, as all other molecular genetic assays. However, its lower limit of detection depends on multiple factors, and is difficult to define. Indeed, different gene fusions can be expressed at different levels, depending on the strength of their transcriptional regulatory regions (promoters, enhancers, etc.). The amount of hybrid mRNA within each sample is also strongly dependent on the size of the biopsies and on the percentage of tumoral cells, both highly variable between different patients.

Because comprehensive DNA and RNA sequencing was not performed in all cases, we cannot be sure that the LD-RT-PCR assay did not miss other cases than the 3 *ALK* translocated tumors, especially cases with *MET* exon 14 skipping mutation. However, this hypothesis is unlikely because this alteration was retrieved in 2.66%, which is consistent with the scientific literature reporting a frequency of around 3%, within the 95% confidence interval, i.e., 1.34–4.72% [34–36]. Likewise, *RET* FISH was not performed routinely and therefore we cannot be certain that some cases were not missed, but a *RET* translocation was retrieved in 0.48%, with a 95% confidence interval (i.e., 0.06–1.74%) within the expected 1% according to the literature [16–18, 37, 38]. Furthermore, our series included a significantly higher proportion of smoking-related tumors than data in the literature, with a *KRAS* mutation retrieved in 35.99% (95% CI = [31.44–40.92%]) compared to 29% according to a 2012–2013 comprehensive French cohort [3]. This could explain the relatively lower frequency of both gene translocations and *EGFR* mutations in our series (not statistically significant though), known not to be associated with never or light smoking. This hypothesis is supported by the fact that the Normandy area of France is known to have a heavy cancer burden due to smoking [39].

In addition, the LD-RT-PCR assay enabled us to determine whether a *MET* mutation retrieved by high-throughput DNA sequencing had a functional skipping effect on exon 14 (Fig. 3B), and therefore whether the patient was eligible for targeted therapy. Indeed, some *MET* mutations are of "unknown significance" according to in silico predictions or data in the literature, and a functional test is sometimes required to assess their biological impact. In our series, 19 samples harbored a *MET* mutation, and LD-RT-PCR was able to distinguish those leading to exon 14 skipping ($n = 11$) and those not ($n = 7$), and therefore to guide the therapy. Results are summarized in Table 4. We reported all the *MET* mutations retrieved in our series, even those which are known not to lead to exon 14 skipping, and which are useful to assess the specificity of LD-RT-PCR (no false positive result). Unfortunately, one sample harboring a *MET* mutation (c.3061T>C) failed LD-RT-PCR analysis.

Regarding the 20 LD-RT-PCR failed tests, 3 factors could explain this failure. First, 5/20 cases were bone biopsies, and therefore underwent a decalcification process known to alter nucleic acids and compromise molecular analysis. Interestingly, not every decalcified tumor failed, as analysis of the remaining 12 bone biopsies was satisfying. However, we were not able to find a satisfying explanation for this. Second, 3/20 cases were cytology samples included in cytoblock after acetic acid fixation according to the routine practice of the laboratory referring the tissue to the molecular platform. Finally, 2/20 cases were cytology slides, and therefore not fixed with formaldehyde. However, for the remaining 10 cases, no satisfying explanation could be found.

Regarding turnaround time, results for LD-RT-PCR were generally available in 3 working days, but of course, since we batched the samples, it could be a bit longer, depending on the date of prescription. For our study, all RNAs were extracted on Monday. The LD-RT-PCR procedure (reverse transcription, hybridization, ligation, PCR amplification and purification) can reasonably be performed in less than one working day, and we repeatedly launched the sequencer on Tuesday evening, depending on its availability. Results were analyzed using our dedicated bioinformatic pipeline in less than half an hour on Wednesday morning.

The next and easy-to-implement improvement in our technique will be to add in the reactional mixture probes able to detect *NTRK* gene family (*NTRK1*, *NTRK2* and *NTRK3*) translocations, suspected to be involved in around 3.3% of "pan-negative" lung cancers and conferring high sensitivity to targeted therapies such as larotrectinib or entrectinib [40–42], as well as *NRG1* translocations, involved in 1–2% of non-small cell lung cancers and sensitive to EGFR and HER3 inhibitors [43–45]. *NTRK* translocations can easily be detected by IHC with a similar process to ALK [46], but given the very few positive cases expected in non-small lung cancer in general, i.e., from 0.1 to 1% [40, 47, 48], it seems unreasonable to perform systematic IHC because of both added costs and tumor tissue loss for each additional staining: in this scenario, LD-RT-PCR becomes particularly relevant. In the future, when new molecular alterations involving translocations or exon skipping mutations predicting response to targeted therapy are described in lung cancer, these could be effortlessly added to the LD-RT-PCR reactional mix at a negligible cost (probe purchase only), without having to change routine practice (no additional consumption of tumor tissue).

We do not deny that other assays are available for high-throughput and comprehensive detection of gene translocations, such as RNA sequencing or Archer© NGS assays (ArcherDX©, Boulder, Colorado). However, these techniques are quite expensive, consume a lot of tumor tissue and require up-to-date sequencing machines, high-level bioinformatic support and highly qualified staff. Furthermore, results are not available in two days, as for LD-RT-PCR. For all these reasons, we believe that these techniques, although useful for some cases, cannot be applied routinely for all newly diagnosed lung carcinomas as we did during one year with LD-RT-PCR. More importantly, LD-RT-PCR seems to be more sensitive than these high-throughput assays, at around 90% compared to 80% when using RNA sequencing for detecting *ALK* translocation [49].

We successfully implemented LD-RT-PCR to detect *ALK*/*ROS1*/*RET* translocation and *MET* exon 14 skipping in the routine molecular analysis of newly diagnosed lung adenocarcinomas for one year. We confirm that this technique is cheap, very fast, simple and easy to implement, both highly sensitive and specific, and easily adaptable for testing new molecular mechanisms involving gene translocations or exon skipping. 19/22 cases harboring *ALK* or *ROS1* translocations were retrieved, with the name of the gene partner, and results were available on average 10 days before FISH confirmation. More importantly, it enabled us to detect 7 cases for which routine practice alone would have missed *RET* translocation or *MET* exon 14 skipping. These cases were all confirmed by other techniques, allowing discussion for prescription of targeted therapy. Additionally, the LD-RT-PCR assay acted as a "functional test" to determine whether *MET* mutations retrieved on high-throughput DNA analysis and of "unknown significance" according to in silico predictions or literature, could lead or not to exon 14 skipping. Increasing numbers of new biomarkers in lung cancer make it difficult to continue sequential systematic testing in all newly diagnosed lung adenocarcinomas, given the scarcity of tumor sample and increasing costs. Therefore, there is a need for "synthetic" approaches such as LD-RT-PCR which can evaluate in a single assay a remarkably high number of biomarkers without consuming more tumor tissue or increasing the price of analysis. Therefore, based on the advantages of LD-RT-PCR, this assay seems to be an excellent alternative to more sophisticated and expensive assays such as RNAseq. We recommend considering it as a cost-effective routine technique for the systematic testing of newly diagnosed lung adenocarcinomas in parallel with ALK/ROS1 IHC and genotyping, and to reserve FISH analysis for discordant cases or "pan-negative" cancers.

Acknowledgements The authors are indebted to Nikki Sabourin-Gibbs (Rouen University Hospital) for assistance with language editing. They would like to thank Dr Clotilde Descarpentries (Lille University Hospital) for genotyping some discordant cases, and Prof. Frédéric Bibeau (Caen University Hospital) for performing *RET* FISH.

Funding This work was mainly funded by *Normandie Valorisation*. NP received additional funding from Boehringer Ingelheim ("Bourse Régionale Profil Onco 2017") and Rouen University Hospital (AOJC no. 2017-12).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. IARC. Globocan 2012. http://globocan.iarc.fr/Pages/fact_sheets_population.aspx.
2. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger KR, Yatabe Y, et al. International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. J Thorac Oncol. 2011;6:244–85.
3. Barlesi F, Mazieres J, Merlio J-P, Debieuvre D, Mosser J, Lena H, et al. Routine molecular profiling of patients with advanced non-small-cell lung cancer: results of a 1-year nationwide programme of the French Cooperative Thoracic Intergroup (IFCT). Lancet. 2016;387:1415–26.
4. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of nonsmall- cell lung cancer to gefitinib. N Engl J Med. 2004;350:2129–39.
5. Soda M, Choi YL, Enomoto M, Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature. 2007;448:561–6.
6. Takeuchi K, Soda M, Togashi Y, Suzuki R, Sakata S, Hatano S, et al. RET, ROS1 and ALK fusions in lung cancer. Nat Med. 2012;18:378–81.
7. Kohno T, Ichikawa H, Totoki Y, Yasuda K, Hiramoto M, Nammo T, et al. KIF5B-RET fusions in lung adenocarcinoma. Nat Med. 2012;18:375–7.
8. Schrock AB, Frampton GM, Suh J, Chalmers ZR, Rosenzweig M, Erlich RL, et al. Characterization of 298 patients with lung cancer harboring MET exon 14 skipping alterations. J Thorac Oncol. 2016;11:1493–502.
9. Camidge DR, Bang Y-J, Kwak EL, Iafrate AJ, Varella-Garcia M, Fox SB, et al. Activity and safety of crizotinib in patients with ALK-positive non-small-cell lung cancer: updated results from a phase 1 study. Lancet Oncol. 2012;13:1011–9.
10. Shaw AT, Ou SH, Bang YJ, Camidge DR, Solomon BJ, Salgia R, et al. Crizotinib in ROS1-rearranged non-small-cell lung cancer. N Engl J Med. 2014;371:1963–71.
11. Velcheti V, Hida T, Reckamp KL, Yang JC, Nokihara H, Sachdev P, et al. Phase 2 study of lenvatinib (LN) in patients (Pts) with RET fusion-positive adenocarcinoma of the lung. Ann Oncol. 2016;27:VI417.
12. Drilon AE, Camidge DR, Ou S-HI, Clarck JW, Socinski MA, Weiss J, et al. Efficacy and safety of crizotinib in patients (pts)

with advanced MET exon 14-altered non-small cell lung cancer (NSCLC). J Clin Oncol. 2016;34:108.
13. Lantuejoul S, Rouquette I, Blons H, Le Stang N, Ilie M, Begueret H, et al. French multicentric validation of ALK rearrangement diagnostic in 547 lung adenocarcinomas. Eur Respir J. 2015;46:207–18.
14. Woo CG, Seo S, Kim SW, Jang SJ, Park KS, Son JY, et al. Differential protein stability and clinical responses of EML4-ALK fusion variants to various ALK inhibitors in advanced ALK-rearranged non-small cell lung cancer. Ann Oncol. 2017;28:791–7.
15. Christopoulos P, Endris V, Bozorgmehr F, Elsayed M, Kirchner M, Ristau J, et al. EML4-ALK fusion variant V3 is a high-risk feature conferring accelerated metastatic spread, early treatment failure and worse overall survival in ALK+ non-small cell lung cancer. Int J Cancer. 2018;142:2589–98.
16. Lipson D, Capelletti M, Yelensky R, Otto G, Parker A, Jarosz M, et al. Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. Nat Med. 2012;18:382–4.
17. Go H, Jung YJ, Kang HW, Park IK, Kang CH, Lee JW, et al. Diagnostic method for the detection of KIF5B-RET transformation in lung adenocarcinoma. Lung Cancer. 2013;82:44–50.
18. Tsuta K, Kohno T, Yoshida A, Shimada Y, Asamura H, Furuta K, et al. RET-rearranged non-small-cell lung carcinoma: a clinicopathological and molecular analysis. Br J Cancer. 2014;100:1571–8.
19. Platt A, Morten J, Ji Q, Elvin P, Womack C, Su X, et al. A retrospective analysis of RET translocation, gene copy number gain and expression in NSCLC patients treated with vandetanib in four randomized phase III studies. BMC Cancer. 2015;15:171.
20. Seo JS, Ju YS, Lee WC, Shin JY, Lee JK, Bleazard T, et al. The transcriptional landscape and mutational profile of lung adenocarcinoma. Genome Res. 2012;22:2109–19.
21. Piton N, Ruminy P, Gravet C, Marchand V, Colasse E, Marguet F, et al. Ligation dependant-RTPCR: a new specific and low-cost technique to detect ALK, ROS and RET rearrangements in lung adenocarcinoma. Lab Invest. 2017;98:371–9.
22. Mareschal S, Ruminy P, Bagacean C, Marchand V, Cornic M, Jais JP, et al. Accurate classification of germinal center B-cell-like/activated B-cell-like diffuse large B-cell lymphoma using a simple and rapid reverse transcriptase-multiplex ligation-dependent probe amplification assay: a CALYM study. J Mol Diagn. 2015;17:273–83.
23. Ruminy P, Marchand V, Buchbinder N, Larson T, Joly B, Penther D, et al. Multiplexed targeted sequencing of recurrent fusion genes in acute leukaemia. Leukemia. 2015;30:757–60.
24. Cho JH, Ku BM, Sun JM, Lee SH, Ahn JS, Park K, et al. KIF5B-MET gene rearrangement with robust antitumor activity in response to crizotinib in lung adenocarcinoma. J Thorac Oncol. 2018;13:e29–e31.
25. Plenker D, Bertrand M, de Langen AJ, Riedel R, Lorenz C, Scheel AH, et al. Structural alterations of MET trigger response to MET kinase inhibition in lung adenocarcinoma patients. Clin Cancer Res. 2018;24:1337–43.
26. Travis WD, Brambilla E, Nicholson AG, Yatabe Y, Austin JHM, Beasley MB, et al. The 2015 World Health Organization Classification of Lung Tumors: impact of genetic, clinical and radiologic advances since the 2004 classification. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2015;10:1243–60.
27. Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, et al. dbSNP: the NCBI database of genetic variation. Nucleic Acids Res. 2001;29:308–11.
28. Landrum MJ, Lee JM, Benson M, Brown GR, Chao C, Chitipiralla S, et al. ClinVar: improving access to variant interpretations and supporting evidence. Nucleic Acids Res. 2018;46:D1062–D1067.
29. Tate GJ, Bamford S, Jubb HC, Sondka Z, Beare MB, Bindal N, et al. COSMIC: the catalogue of somatic mutations in cancer. Nucleic Acids Res. 2019;47:D941–D947.
30. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discovery. 2012;2:401–4.
31. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer O, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci. Signal. 2013;6:pl1.
32. Vaser R, Adusumalli S, Leng SN, Sikic M, Ng PC. SIFT missense predictions for genomes. Nat Protocols. 2016;11:1–9.
33. Schwarz J, Cooper D, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. Nat Methods. 2014;11:361–2.
34. Frampton GM, Ali SM, Rosenzweig M, Chmielecki J, Lu X, Bauer TM, et al. Activation of MET via diverse exon 14 splicing alterations occurs in multiple tumor types and confers clinical sensitivity to MET inhibitors. Cancer Discov. 2015;5:850–9.
35. Awad MM, Oxnard GR, Jackman DM, Savukoski DO, Hall D. Shivdasani et al. MET exon 14 mutations in non-small-cell lung cancer are associated with advanced age and stage-dependent MET genomic amplification and c-MET overexpression. J Clin Oncol. 2016;34:721–30.
36. Tong JH, Yeung SF, Chan AWH, Chung LY, Chau SL, Lung RWM, et al. MET amplification and exon 14 splice site mutation define unique molecular subgroups of non-small cell lung carcinoma with poor prognosis. Clin Cancer Res. 2016;22:3048–56.
37. Ju YS, Lee W-C, Shin J-Y, Lee S, Bleazard T, Won J-K et al. A transforming KIF5B and RET gene fusion in lung adenocarcinoma revealed from whole-genome and transcriptome sequencing. Genome Res. 2012;22:436–45.
38. Varella-Garcia M, Xu LG, Mahale S, Berge EM, Bennati C, Aisner AL et al. RET rearrangements detected by FISH in "pan-negative" lung adenocarcinoma. J Clin Oncol 2013;31(suppl; abstr 8024).
39. Santé Publique France. Bulletin de santé publique Normandie. 2019. http://invs.santepubliquefrance.fr/content/download/153167/559371/version/44/file/bsp_tabac_normandie_janvier_2019.pdf.
40. Vaishnavi A, Capelletti M, Le AT, Kako S, Butaney M, Ercan D, et al. Oncogenic and drug-sensitive NTRK1 rearrangements in lung cancer. Nat Med. 2013;19:1469–72.
41. Drilon A, Siena S, Ou SI, Patel M, Ahn MJ, Lee J, et al. Safety and antitumor activity of the multitargeted pan-TRK, ROS1, and ALK inhibitor entrectinib: combined results from two phase I trials (ALKA-372-001 and STARTRK-1). Cancer Discov. 2017;7:400–9.
42. Farago A, Kummar S, Ibabekci S, Corsi-Travali S, Cruickshank S, Cox M, et al. P1.13-40 rapid, robust and durable responses to Larotrectinib in patients with TRK fusion non-small cell lung Cancer. J Thorac Oncol. 2018;13:S597–8.
43. Muscarella LA, Rossi A. NRG1: a cinderella fusion in lung cancer? Lung Cancer Manag. 2017;6:121–3.
44. Kim HS, Han JY, Shin DH, Lim KY, Lee GK, Kim JY, et al. EGFR and HER3 signaling blockade in invasive mucinous lung adenocarcinoma harboring an NRG1 fusion. Lung Cancer. 2018;124:71–5.
45. Shin DH, Lee D, Hong DW, Hong SH, Hwang JA, Lee BI, et al. Oncogenic function and clinical implications of SLC3A2-NRG1 fusion in invasive mucinous adenocarcinoma of the lung. Oncotarget. 2016;7:69450–65.
46. Hechtman JF, Benayed R, Hyman DM, Drilon A, Zehir A, Frosina D, et al. Pan-Trk immunohistochemistry is an efficient and reliable screen for the detection of NTRK fusions. Am J Surg Pathol. 2017;41:1547–51.

47. Vaishnavi A, Le AT, Doebele RC. TRKing down an old oncogene in a new era of targeted therapy. Cancer Discov. 2015;5:25–34.
48. Farago AF, Le LP, Zheng Z, Muzikansky A, Drilon A, Patel M, et al. Durable clinical response to entrectinib in NTRK1-rearranged non-small cell lung cancer. J Thorac Oncol. 2015;10:1670–4.
49. McLeer-Florin A, Duruisseaux M, Pinsolle J, Dubourd S, Mondet J, Phillips Houlbracq M, et al. ALK fusion variants detection by targeted RNA-next generation sequencing and clinical responses to crizotinib in ALK-positive non-small cell lung cancer. Lung Cancer. 2018;116:15–24.
50. https://www.hcp.novartis.com/globalassets/migration-root/hcp/products2/tabrecta/pdf/tabrecta-hcp-testing-brochure.pdf.