Short Communication

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Personalized Medicine

RNA-based next generation sequencing in non-small-cell lung cancer in a routine setting: an experience from an Italian referral center

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Aim: *ALK*, *ROS1*, *NTRK* and *RET* gene fusions and *MET* exon 14 skipping alterations represent novel predictive biomarkers for advanced non-small-cell lung cancer (NSCLC). Therefore, testing patients for these genetic variants is crucial for choosing the best selective treatment. Over the last couple of decades, NGS platforms have emerged as an extremely useful tool for detecting these variants. **Materials & methods:** In the present study, we report our NGS molecular records produced during a year of diagnostic activity. **Results:** Overall, our in-house developed NGS workflow successfully analyzed n = 116/131 (88.5%) NSCLC samples. Of these, eight (6.8%) and five (4.3%) out of 116 patients harbored *ALK* and *RET* gene rearrangements, respectively: one case harbored *ROS1* gene fusion (0.7%). **Conclusion:** Our results highlight that an RNA-based NGS analysis can reliably detect gene fusion alterations, thereby playing a pivotal role in the management of NSCLC patients.

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Lung cancer still remains the leading cause of cancer mortality worldwide. A staggering 2,206,771 new diagnoses occurred in 2020 [1]. Remarkably, approximately 84% of lung cancer patients are diagnosed with advanced non-small-cell lung cancer (NSCLC) a reality that severely affects the clinical and financial management of newly diagnosed NSCLC patients [2,3].. Major advances in lung cancer treatment have been made in recent years, thanks to the advent of personalized medicine. Indeed, an increasing number of predictive biomarkers have been approved by national and international regulatory agencies in order to select the best therapeutic options for NSCLC patients [4]. Among these biomarkers are hot spot mutations in *EGFR*, B-Raf proto-oncogene (*BRAF*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*) exon 2 p.G12C, MET proto-oncogene, receptor tyrosine kinase (*MET*)

exon 14 skipping. A recent study has demonstrated that targeting these DNA-based alterations with tyrosine kinase inhibitors (TKIs) improves clinical outcomes in NSCLC patients [5]. In addition to DNA-based mutations, gene fusions also represent therapeutically targetable biomarkers in lung cancer. Indeed, although they occur less frequently in NSCLC, they must also be taken into account to give these patients the opportunity to receive targeted treatments [6].

Overall, the shift from conventional to biomarker-based personalized approaches in lung cancer has borne fruit and inspired further research in the field. Indeed, besides the well-established efficacy of specific TKIs in improving progression free survival and overall survival of NSCLC patients [7]. Immune-check point inhibitors have also shown promising results against yet another cancer biomarker, namely PD-L1 [8,9].

Thus, assessing the comprehensive molecular landscape of advanced stage NSCLC patients is paramount in the treatment decision making process. Although, different diagnostic approaches are currently available for gene fusion analysis [6] in a vast majority of advanced NSCLC patients small tissue samples (histological biopsies or cytological specimens) are the only available material for morph-molecular analysis [10]. Accordingly, conventional single gene testing technology may be inadequate to cover the wide and ever evolving landscape of druggable biomarkers. In this scenario, the use of next generation sequencing (NGS) for the development of diagnostic algorithm of advanced stage NSCLC is gaining increasing popularity in routine clinical laboratory practice. Indeed, unlike conventional approaches, DNA- and RNA based NGS technologies can simultaneously analyze all clinically relevant biomarkers, thus lowering the overall time and costs involved in the sequencing process [11].

In this setting, our Molecular Predictive Pathology Laboratory at the Department of Public Health of the University of Naples Federico II has long adopted NGS technology to assesses clinically relevant biomarkers in different solid tumors [12,13].

Here, we review our NGS molecular records for anaplastic lymphoma kinase (*ALK*), ROS proto-oncogene 1 (*ROS1*), neurotrophic tyrosine receptor kinase (*NTRK*), rearranged during transfection (*RET*) gene fusions and MET proto-oncogene, receptor tyrosine kinase (*MET*) exon 14 skipping alterations obtained during 1 year of diagnostic routine practice.

Materials & methods

Records of previously tested *ALK*, *ROS1*, *RET* and *NTRK* rearrangements [11] and *MET* exon 14 skipping mutations from n = 131, NSCLC patients were retrieved from our internal archive. All molecular tests were carried out with our in-house upfront RNA-based NGS assay from December 2020 to December 2021. In particular, RNA extraction from formalin-fixed paraffin embedded (FFPE) tissues and cytological stained smears was performed with the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After RNA retrotranscription, cDNA was analyzed on the Ion S5™ System (Thermo Fisher Scientific, MA, USA). Libraries were constructed and purified on the Ion Chef Instrument (Thermo Fisher Scientifics) according to the manufacturer's instructions. Briefly, 15 µl of cDNA was dispensed on the Ion Code plates and amplified with Ion AmpliSeq DL8 (Thermo Fisher Scientifics). Then, cDNA was amplified with 26 cycles; instead, the library was re-amplified with four PCR cycles after barcoding, under the thermal conditions recommended by the manufacturer. Purified libraries from RNA samples were diluted to 60 pM and pooled together. Next templates for NGS analysis were prepared with the S5 510–520–530 Kit-Chef (Thermo Fisher Scientifics). After preparation, they were loaded onto a 520 chip and sequenced on the S5 NGS platform (Thermo Fisher Scientifics). A quality score $\geq$20 was required to identify *ALK*, *ROS1*, *RET* and *NTRK* gene fusions and *MET* exon 14 skipping alterations [11]. In addition, BAM files were uploaded on Ion Reporter Torrent Suite version 5.18.0.1 with a dedicated workflow optimized for fusion detection.

For immunohistochemistry (IHC) analysis, 4-µm thick FFPE tissue sections were processed (VENTANA-Roche ALK – D5F3 CDx Assay) according to the manufacturer's instructions. The analysis was performed by the automated Benchmark Ultra (Roche-Ventana Medical System, AZ, USA) platform. The IHC signal was evaluated by an expert pathologist. Slides with a diffuse, strong cytoplasmatic signal, which was confirmed by the positive control, were considered positive for *ALK* rearrangements. In addition, a confirmatory IHC analysis for *ROS1*, performed on all 4 µm-thick FFPE tissue sections, was done by adopting clone D4D6 (Cell signaling technology, MA, USA]. In both IHC analyses, a tyramide-based amplification phase in combination with the polymeric step (OptiView Ventana Medical Systems) was used. Finally, a dichotomous scoring system (positive or negative) was adopted to classify each case. All IHC results were compared with NGS positive results.

Table 1. Summary of the clinical features of analyzed patients and next generation sequencing technical parameters.

Neoplastic cell percentage (%)	42.0%
Sex	
Female	51.0 (39.2%)
Male	79.0 (60.8%)
Sample Type	
Histological	87.0 (67.0%)
Cytological	43.0 (33.0%)
Average Reads	469710.1
Average Mapped Reads	433786.0
Average percentage of reads on target	95.2%
Average of reads per amplicon	4568.0
Average uniformity of coverage	95.8%

Table 2. Detected fusions.

ID sample	Morphological diagnosis	NGS result	IR read count
2	*ADC*	*ALK* (ex20) / *EML4* (ex13)	10251
5	*ADC- signet ring cells*	*ALK* (ex20) / *EML4* (ex13)	8238
17	*ADC*	*ALK* (ex20) / *EML4* (ex6)	601
32	*ADC*	*ALK* (ex20) / Unknown	41,17587
33	*ADC*	*RET* / unknown partner	86617,15207
45	*ADC*	*RET* (ex12)/ *KIF5B* (ex15)	494
56	*ADC*	*ALK* (ex20) / *EML4* (ex6)	2549
68	*ADC*	*ALK* (ex20) / unknown partner	149,5199
69	*ADC*	*ALK* (ex20) / *EML4* (ex20)	21789
84	*ADC*	*RET* / unknown partner	41279,12019
94	*ADC*	*RET* / unknown partner	21336,35917
97	*ADC*	*ALK* (ex20) / *EML4* (ex13)	3799
111	*ADC*	*RET* / unknown partner	21922,35005
131	*ADC*	*CD74 (ex6) – ROS1 (ex34)*	2208

ALK: Anaplastic lymphoma kinase; *CD74*: CD74 molecule; *EML4*: Echinoderm microtubule associated protein like 4; *KIF5B*: Kinesin family member 5B; NGS: Next generation sequencing; *RET*: Rearranged during transfection; *ROS1*: ROS proto-oncogene 1.

Results

The study included 131 samples from advanced NSCLC patients with a median age of 65.6 years (ranging from 24.0 to 92.0). Of these, n = 51/131 (38,9%) were females and n = 80/131 (61,1%) were males. In brief, n = 88/131 (67.2%) were histological samples; in particular, n = 55/88 (62.5%) were biopsies and n = 33/88 (37.5%) were surgical resections. The remaining 43 samples (32.8%) were cytological specimens from fine needle aspiration biopsy procedures; in particular n = 28/43 (65.1%) were cell-blocks and n = 15/43 (34.9%) were stained smears. Median value of neoplastic cell percentage was 42.0% (ranging from 10.0 to 80.0%).

Overall, our in-house developed NGS workflow successfully analyzed n = 116/131 (88.5%) NSCLC samples. The inadequate cases resulted from low quality RNA. NGS analysis produced the following parameters: a median number of reads of 467738.1 (ranging from 23185.0 to 1280795.0), a median number of read lengths of 100.8 bp (ranging from 48 to 125 bp), a median number of mapped reads of 432111 (ranging from 18708.0 to 1252125.0), an average percentage of reads on target of 95.2% (ranging from 38.7 to 99.9%), an average of reads per amplicon of 4551 (ranging from 197.0 to 13237.0), and a uniformity of coverage of 95.9% (ranging from 79.1 to 99.2%). More details are reported in Table 1 and Supplementary Table 1. Notably, NGS correctly analyzed 102/116 (87.9%) negative cases and 14/116 (12.1%) positive cases (Figure 1). In particular, it successfully detected *ALK* and *RET* rearrangements in eight (6.8%) and five (4.3%) out of the 116 patients; moreover, it was even able to identify a single case of *ROS1* gene fusion (0.7%) (Table 2) (Figure 1). As for our comparative analyses, eight out of 14 (57.1%) mutated cases displayed the same molecular results. Interestingly, *ALK* and *ROS1* NGS-positive cases were further confirmed by IHC. Unfortunately, no additional material was available to perform fluorescent *in situ* hybridization (FISH) in *RET*- and *ROS1*- rearranged patients. (Figure 1)

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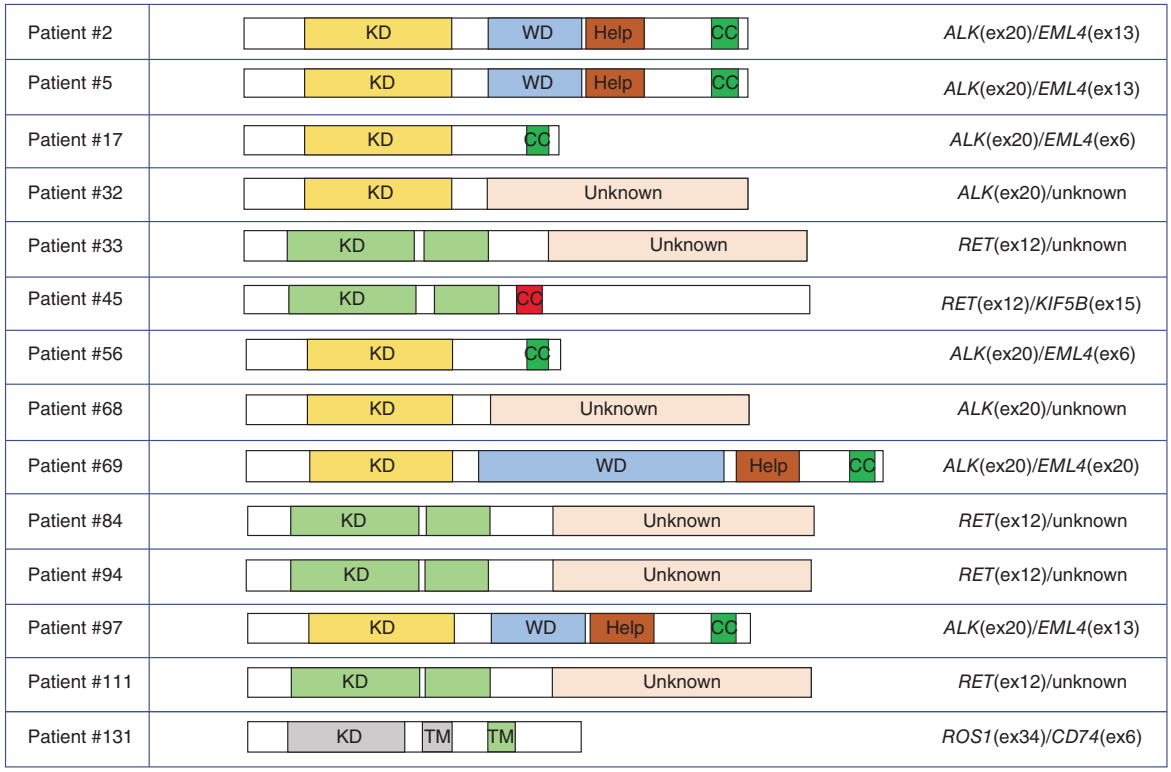

Figure 1. *ALK*, *ROS1* and *RET* gene fusions.
ALK: Anaplastic lymphoma kinase; CC: Coiled coil; *CD74*: CD74 molecule; *EML4*: Echinoderm microtubule associated protein like 4; HELP: Hydrophobic motif in EML proteins; KD: Kinase domain; *KIF5B*: Kinesin Family Member 5B; *RET*: Rearranged during transfection; *ROS1*: ROS proto-oncogene 1; TM: Transmembrane; WD: Tryptophan-aspartic acid domain.

Discussion

This study highlights the feasibility of using an RNA-based NGS approach to identify clinically relevant gene fusions in advanced NSCLC patients. In particular, our laboratory-developed assay panel, namely SiRe fusion [13], achieved an overall success rate as high as 88.4% in both histological and cytological specimens. In particular, it was able to detect positive *ALK* rearrangements in 8/116 cases (6.8%) and positive *RET* rearrangements in 5/116 (4.3%) cases. In addition, it successfully identified *ROS1* gene fusion in one case (0.7%). The identification of these fusion genes is key to selecting NSCLC patients eligible for TKI treatment [14,15]. Indeed, these treatments have proven highly successful in improving the clinical outcomes of patients. We do realize that there were some discrepancies between our results and previous findings. However, we hypothesize that such inconsistencies were not due to sequencing errors but to the low number of patients enrolled in our study. For this same reason, we were not able to detect *MET* exon 14 skipping alterations. Notably, all *ALK* and *ROS1* rearranged cases were further confirmed by IHC.

Despite the low number of cases analyzed, our results, along with previous findings accumulated over the past years, fully reflect the general agreement among molecular scientists on the advantages of using NGS technology in clinical laboratories. Indeed, molecular evaluation of gene rearrangements has now become an integral part of the comprehensive molecular analysis for the clinical management of advanced NSCLC patients [7]. Currently, different methodologies can be employed to identify these genomic alterations [6]. However, all of them come with advantages and disadvantages. As of today, the FISH approach is considered the gold standard assay to detect gene fusions. However, its full application in routine clinical practice is oftentimes hindered by its high costs, low specificity, high inter-observed variability and long turnaround time [16]. A valid alternative to FISH is either IHC or immunocytochemistry. Both approaches are widely used in anatomic pathology laboratories. However, in spite of their popularity, cost–effectiveness and short turnaround time, their use is limited by their low sensitivity and specificity for specific gene fusions, particularly, *ROS1* and *RET* gene rearrangements [17,18]. The same can be said

for RT-PCR. Although, a major advantage of this molecular approach is its high sensitivity, this approach can only detect well-known gene fusions and has limited multiplexing capability [6]. Generally, all the above methodologies share a major common shortcoming: they are unable to analyze multiple genomic alterations in a single analysis.

The past two decades have witnessed a raft of efforts to address this challenge. A major breakthrough has been the development of NGS technologies, otherwise known as high-throughput sequencing technologies. What makes NGS assays more powerful than conventional approaches, especially for NSCLC patients, is its ability to analyze multiple clinically relevant biomarkers even in scant tissue samples. Not surprisingly, its implementation has become indispensable in diagnostic practice especially for the detection of gene rearrangements. Noteworthy, it can analyze not only DNA but also RNA samples. A major strength of RNA-based gene rearrangement analysis, compared with DNA-based analysis, lies in its ability to analyze transcripts rather than large intronic regions [19]. In addition, an RNA-based NGS approach enables laboratory clinicians to spend less time on testing activities, thereby reducing the overall cost of testing per patient, compared with PCR-based methods [20]. However, since RNA is less stable than DNA, special attention must be devoted to all the pre-analytical steps to avoid false negative results.

In conclusion, our molecular analysis, carried out on a retrospective series of advanced NSCLC patients, has highlighted the applicability and efficiency of a custom RNA-based NGS approach to analyze gene fusion rearrangements in advanced stage NSCLC patients. Indeed, adopting an RNA-based NGS approach in routine clinical practice would be instrumental in managing these patients, especially for cases with extremely scant tumor material. Further studies are warranted to optimize RNA-based NGS approaches not only on tumor tissues but also on liquid biopsies.

Summary points

- *ALK*, *ROS1*, *NTRK* and *RET* gene fusions, as well as *MET* exon 14 skipping alterations, represent novel predictive biomarkers for advanced non-small-cell lung cancer (NSCLC).
- We reported our referral laboratory experience in gene fusion molecular analysis by RNA-next generation sequencing (NGS) in a retrospective series of advanced stage NSCLC patients referred to our clinic from December 2020 to December 2021.
- Our in-house RNA-based NGS assay correctly analyzed 88.4% of gene rearrangements. These findings suggest that NGS platforms are instrumental in detecting clinically relevant gene fusions in NSCLC patients, thereby facilitating the selection of patients for targeted treatments, especially for cases with scant tissue material.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/pme-2022-0020

Author contributions

Conceptualization: U Malapelle; methodology: all authors; software: all authors; validation: all authors; formal analysis: all authors; investigation: all authors; resources: all authors; data curation: all authors; writing – original draft preparation: C De Luca, F Pepe, P Pisapia, U Malapelle; writing – review & editing: all authors; visualization: all authors; supervision: G Troncone, U Malapelle; project administration, G Troncone, U Malapelle; funding acquisition: G Troncone.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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