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Original article

# RNA-Based Next-Generation Sequencing in Non-Small Cell Lung Cancer patients: data from Campania, Italy

Pasquale Pisapia[1], Antonino Iaccarino[1], Caterina De Luca[1], Francesco Pepe[1], Gianluca Russo[1], Mariantonia Nacchio[1], Francesca Ambrosio[2], Roberto Bianco[3], Severo Campione[4], Alessandro Caputo[5], Pietro Carotenuto[6,7], Antonio D'Antonio[8], Maria D'Armiento[1], Vincenzo Damiano[3], Bruno Daniele[9], Giovanni De Chiara[10], Marco De Felice[11], Luigi Della Gravara[12], Teresa Fabozzi[9], Salvatore Feliciano[13], Cesare Gridelli[14], Elia Guadagno[4], Gennaro Ilardi[15], Davide Leopardo[16], Annamaria Libroia[17], Paolo Maione[14], Floriana Morgillo[12], Jessica Orefice[9], Luigi Panico[18], Danilo Rocco[19], Alberto Servetto[3], Silvia Varricchio[15], Pio Zeppa[5], Elena Vigliar[1], Claudio Bellevicine[1], Giancarlo Troncone[1], Umberto Malapelle[1]

*[1] Department of Public Health, University of Naples Federico II, Naples, Italy; [2] Oncology Unit, A.O.R.N. Cardarelli, Hospital Antonio Cardarelli, Naples, Italy; [3] Department of Clinical Medicine and Surgery, University of Naples Federico II, Naples, Italy; [4] Pathology Unit, A.O.R.N. Cardarelli, Hospital Antonio Cardarelli, Naples, Italy; [5] Department of Pathology, University Hospital of Salerno, Salerno, Italy; [6] Department of Translational Medical Science, Medical Genetics, University of Naples Federico II, Naples, Italy; [7] TIGEM, Telethon Institute of Genetics and Medicine, Pozzuoli, Naples, Italy; [8] Pathology Unit, Ospedale del Mare, Naples, Italy; [9] Oncology Unit, Ospedale del Mare, Naples, Italy; [10] Division of Pathology, "S.G. Moscati" Hospital, Avellino, Italy; [11] Medical Oncology Unit, Ospedale Ave Gratia Plena, Piedimonte Matese, Caserta, Italy; [12] Department of Precision Medicine, Università degli Studi della Campania "Luigi Vanvitelli", Naples, Italy; [13] Medical Oncology Unit, Ospedale Ave Gratia Plena, San Felice a Cancello, ASL Caserta, Caserta, Italy; [14] Division of Medical Oncology, "S.G. Moscati" Hospital, Avellino, Italy; [15] Department of Advanced Biomedical Sciences, University of Naples Federico II, Naples, Italy; [16] Department of Oncology, A.O.R.N. Sant'Anna e San Sebastiano, Caserta, Italy; [17] Oncology Unit, "Andrea Tortora" Hospital, ASL Salerno, Pagani, Italy; [18] Department of Pathology, A.O.R.N. dei Colli Monaldi, Naples, Italy; [19] Department of Pulmonary Oncology, A.O.R.N. dei Colli Monaldi, Naples, Italy*

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**Correspondence**
Giancarlo Troncone
E-mail: giancarlo.troncone@unina.it

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## Summary

**Objective.** *ALK*, *ROS1*, *NTRK*, and *RET* gene fusions and *MET* exon 14 skipping alterations represent fundamental predictive biomarkers for advanced non-small cell lung cancer (NSCLC) patients to ensure the best treatment choice. In this scenario, RNA-based NGS approach has emerged as an extremely useful tool for detecting these alterations. In this study, we report our NGS molecular records on *ALK*, *ROS1*, *NTRK*, and *RET* gene fusions and *MET* exon 14 skipping alterations detected by using a narrow RNA-based NGS panel, namely SiRe fusion.

**Methods.** We retrospectively reviewed data on 201 advanced stage NSCLC patients who were referred to our laboratory for RNA-based molecular evaluation of *ALK*, *ROS1*, *RET*, *NTRK* gene rearrangements as well as *MET* exon 14 skipping.

**Results.** Overall, 23 (11.4%) positive cases were retrieved. Regarding molecular assessment, 11 (5.5%), 2 (1.0%), 9 (4.5%), and 1 (0.5%) out of 201 harbored an *ALK*, *ROS1*, *RET* gene rearrangement, or *MET* exon 14 skipping, respectively.

**Conclusions.** In this study, we provide real-world experience on RNA-based NGS analysis in patients with advanced stage NSCLC.

**Keywords:** NSCLC, NGS, predictive molecular pathology, molecular oncology, RNA-based biomarkers

## Introduction

Non-small cell lung cancer (NSCLC) represents the leading cause of cancer mortality worldwide [1]. Unfortunately, the vast majority of patients

(more than 80%) are diagnosed in an advanced stage of disease, with a significant impact on treatment decision making and overall outcome [2]. However, several efforts have been spent to improve the quality of life, progression free survival and overall survival of these patients through personalized medicine [3]. As a consequence, the number of approved predictive biomarkers that must be tested has rapidly increased [4-6]. Among these are point mutations and indels in Epidermal Growth Factor Receptor (*EGFR*), Erb-B2 Receptor Tyrosine Kinase 2 (*ERBB2*), V-Raf Murine Sarcoma Viral Oncogene Homolog B1 (*BRAF*) exon 15 p.V600E, Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*) exon 2 p.G12C, that can be investigated at a DNA-level [7]. Another important biomarker is immunohistochemical/immunocytochemical evaluation of the expression levels of Programmed Death-Ligand 1 (PD-L1) for immune-checkpoint inhibitors (ICIs) administration [8-11]. Beyond DNA-based biomarkers and PD-L1 expression evaluation, another group of biomarkers is represented by Anaplastic Lymphoma Kinase (*ALK*), ROS Proto-Oncogene 1 Receptor Tyrosine Kinase (*ROS1*), Neurotrophic Receptor Tyrosine Kinase (*NTRK*), REarranged during Transfection (*RET*) gene rearrangements, and MET Proto-Oncogene, Receptor Tyrosine Kinase (*MET*) exon 14 skipping, which can be analyzed on RNA [12]. Despite the increasing number of biomarkers, in the vast majority of advanced NSCLC patients only small tissue samples are available for morph-molecular analysis [13]. In this scenario, next generation sequencing (NGS), able to analyze all biomarkers at lower costs and turnaround time respect to single-gene testing approaches [14], represents a valid solution. In our referral Molecular Predictive Pathology Laboratory at the Department of Public Health of the University of Naples Federico II, we routinely perform IHC/ICC to evaluate PD-L1 expression [10,11], and we employ a complementary DNA-based and RNA-based NGS approaches to evaluate genomic alterations useful for target therapy administration in advanced stage NSCLC patients [15-18].

Here, we retrospectively evaluated data collected from our archives on advanced stage NSCLC patients tested by our NGS RNA-based approach who were referred to our laboratory for the evaluation of *ALK*, *ROS1*, *NTRK*, *RET* gene rearrangements and *MET* exon 14 skipping during two years of diagnostic routine practice. In addition, in a subset of patients, we were also able to retrieve information about patients' medical treatments.

## Materials and methods

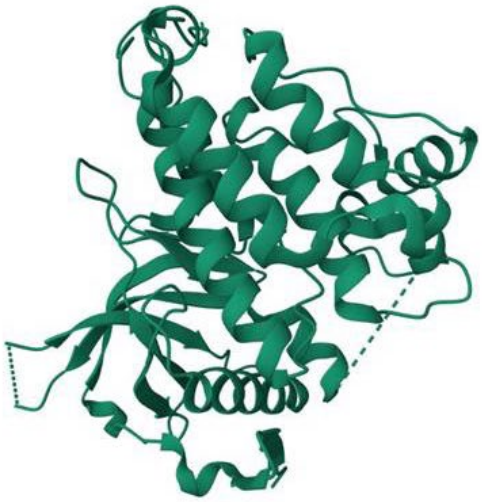
### Study design

We retrospectively retrieved from our electronic archives advanced stage NSCLC cases tested by our DNA- and RNA-based NGS approach as well as PD-L1 expression level evaluation referred to our laboratory from December 2020 to December 2022. Data regarding sex, median age, sample type and subtype, and diagnosis was also retrieved for *ALK*, *ROS1*, *RET*, *NTRK* rearranged and *MET* exon 14 skipping patients (Figs. 1-4, Tabs. I-II). In addition, for a subset of these patients, information related to the duration of the first or other line treatments, or until the loss of data for any causes, was also gathered.

Written informed consent was obtained from all patients, in accordance with the general authorization to process personal data for scientific research purposes from "The Italian Data Protection Authority" (http://www.garanteprivacy.it/web/guest/home/docweb/-/docwebdisplay/export/2485392). All information regarding human material was managed using anonymous numerical codes, and all samples were handled in compliance with the Declaration of Helsinki (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/).

### Molecular testing

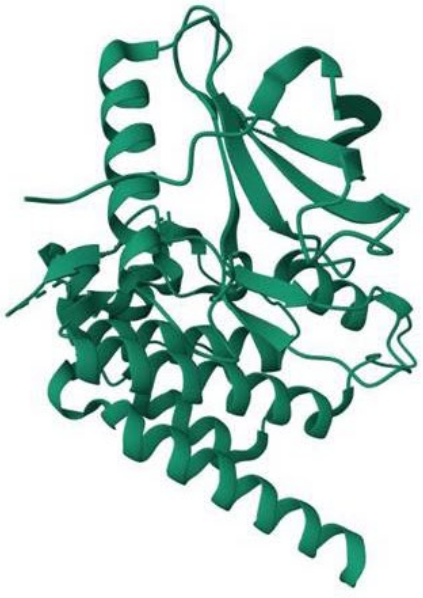
Molecular testing was carried out as previously described [15-18]. Briefly, DNA and RNA were extracted from formalin-fixed paraffin embedded (FFPE) tissues and cytological smears by using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Regarding RNA analysis, retro-transcription was performed. DNA and cDNA were analyzed on an Ion S5™ System (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were constructed and purified on the Ion Chef Instrument (Thermo Fisher Scientifics, Waltham, MA, USA) according to the manufacturer's instructions. After preparation, they were loaded onto a 520 chip and sequenced on the S5 NGS platform (Thermo Fisher Scientifics). Overall, DNA-based NGS analysis was performed by using our narrow NGS panel, namely, SiRe®, which is able to cover multiple hotspot gene alterations in seven genes (*EGFR*, *KRAS*, *BRAF*, Neuroblastoma RAS Viral Oncogene Homolog [*NRAS*], KIT Proto-Oncogene, Receptor Tyrosine Kinase [*KIT*], Platelet Derived Growth Factor Receptor Alpha [*PDGFRα*], and Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha [*PIK3CA*]), as previously described [15,16]. RNA-based NGS analy-



| | *ALK* |
|---|---|
| Total (%) | 11 (100.0) |
| Sex (%) | M: 1 (9.1)<br>F: 10 (90.9) |
| Median Age (range) | 56.3 y (35.0 – 76.0 y) |
| Sample type (n; %)<br>- subtype (n; %) | Histological (8, 72.7)<br>- Biopsy (7, 87.5)<br>- Resection (1, 12.5)<br>Cytological (3, 27.3)<br>- Cell block (3, 100.0) |
| Diagnosis (n, %) | NSCLC ADC (1, 9.1)<br>NSCLC favor ADC (10, 90.9) |
| Alteration (n, %) | *EML4*(13)::*ALK*(20) (4, 36.3)*<br>*EML4*(20)::*ALK*(20) (2, 18.2)<br>*EML4*(6)::*ALK*(20) (3, 27.3)**<br>unknown::*ALK*(20) (2, 18.2)*** |

* in two instances with concomitant PD-L1 1-49% expression; in one instance with concomitant PD-L1 ≥50% expression.
** in one instance with concomitant PD-L1 1-49% expression; in one instance with concomitant PD-L1 ≥50% expression.
*** in one instance with concomitant PD-L1 1-49% expression.

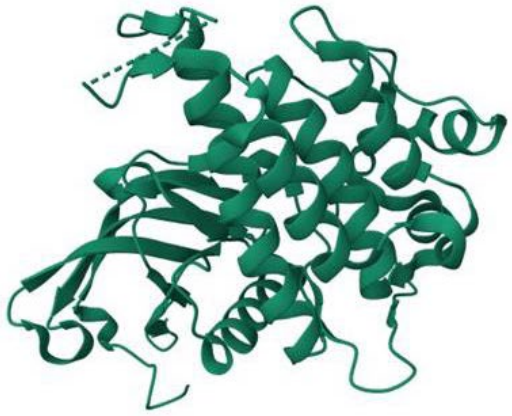
**Figure 1.** *ALK* rearranged cases with clinical variables. This figure was created by using Protein Data Bank (PDB) (https://www.rcsb.org/).
ADC: adenocarcinoma; *ALK*: Anaplastic Lymphoma Receptor Tyrosine Kinase; *EML4*: Echinoderm Microtubule-Associated Protein-Like 4; F: female; M: male; MET: MET Proto-Oncogene, Receptor Tyrosine Kinase; n: number; PD-L1: Programmed death-ligand 1.



| | *ROS1* |
|---|---|
| Total (%) | 2 (100.0) |
| Sex (%) | M: 1 (50.0)<br>F: 1 (50.0) |
| Median Age (range) | 28.5 y (24.0 – 33.0 y) |
| Sample type (n; %)<br>- subtype (n; %) | Histological (2, 100.0)<br>- Biopsy (2, 100.0) |
| Diagnosis (n, %) | NSCLC favor ADC (2, 100.0) |
| Alteration (n, %) | *CD74*(6)::*ROS1*(34) (2, 100.0)**** |

**** in one instance with concomitant PD-L1 ≥50% expression

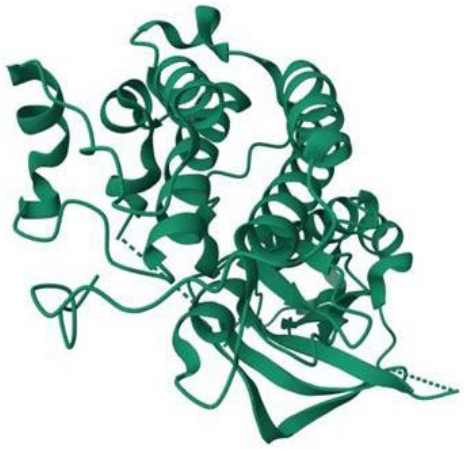
**Figure 2.** *ROS1* rearranged cases with clinical variables. This figure was created by using Protein Data Bank (PDB) (https://www.rcsb.org/).
ADC: adenocarcinoma; *CD74*: CD74 Molecule, Major Histocompatibility Complex, Class II Invariant Chain; F: female; M: male; n: number; PD-L1: Programmed death-ligand 1; *ROS1*: ROS Proto-Oncogene 1, Receptor Tyrosine Kinase.



| | *RET* |
|---|---|
| Total (%) | 9 (100.0) |
| Sex (%) | M: 4 (44.4)<br>F: 5 (55.6) |
| Median Age (range) | 64.8 y (47.0 – 79.0 y) |
| Sample type (n; %)<br>- subtype (n; %) | Histological (5, 55.6)<br>- Biopsy (3, 60.0)<br>- Resection (2, 40.0)<br>Cytological (4, 44.4)<br>- Cell block (2, 50.0)<br>- Smear (2, 50.0) |
| Diagnosis (n, %) | NSCLC favor ADC (8, 88.9)<br>NSCLC ADC + SqCC (1, 11.1) |
| Alteration (n, %) | *CCDC6*(1)::*RET*(12) (1, 11.1)<br>*KIF5B*(15)::*RET*(12) (5, 55.6)*****<br>unknown::*RET*(12) (3, 33.3)****** |

***** in one instance with concomitant *PIK3CA* exon 9 p.E545K; in two instances with concomitant PD-L1 1-49% expression.
****** in one instance with concomitant *KRAS* exon 2 p.G12D; in one instance with concomitant PD-L1 1-49% expression.

**Figure 3.** *RET* rearranged cases with clinical variables. This figure was created by using Protein Data Bank (PDB) (https://www.rcsb.org/).
ADC: adenocarcinoma; F: female; *KIF5B*: Kinesin Family Member 5B; *KRAS*: Kirsten Rat Sarcoma Viral Oncogene Homolog; M: male; n: number; PD-L1: Programmed death-ligand 1; PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; RET: Rearranged During Transfection; SqCC: squamous cell carcinoma.



| | *MET* |
|---|---|
| Total (%) | 1 (100.0) |
| Sex (%) | F: 1 (100.0) |
| Median Age (range) | 72.0 y (72.0 y) |
| Sample type (n; %)<br>- subtype (n; %) | Histological (1, 100.0)<br>- Biopsy (1, 100.0) |
| Diagnosis (n, %) | NSCLC NOS (1, 100.0) |
| Alteration (n, %) | *MET* exon 14 skipping (1, 100.0)******* |

******* with concomitant PD-L1 ≥50% expression.

**Figure 4.** *MET* exon 14 skipping case with clinical variables. This figure was created by using Protein Data Bank (PDB) (https://www.rcsb.org/).
F: female; *MET*: MET Proto-Oncogene, Receptor Tyrosine Kinase; n: number; NOS: not otherwise specified; PD-L1: Programmed death-ligand 1.

**Table I.** Clinical and molecular findings of the study population.

| | Global |
|---|---|
| Total (%) | 201 (100.0) |
| Adequacy rate (n, %) | Adequate (200, 99.5)<br>Inadequate (1, 0.5) |
| RNA-based molecular alteration (n, %) | Negative (177, 88.5)<br>Positive (23, 11.5) |
| RNA-based molecular alteration type (n, %) | *ALK* (11, 5.5)<br>*ROS1* (2, 1.0)<br>*RET* (9, 4.5)<br>*MET* exon 14 skipping (1, 0.5) |
| Sex (%) | M: 6 (26.1)<br>F: 17 (73.9) |
| Median Age (range) | 57.9 y (24.0 – 79.0 y) |
| Sample type (n; %)<br>- subtype (n; %) | Histological (16, 69.6)<br>- Biopsy (13, 81.3)<br>- Resection (3, 18.7)<br>Cytological (7, 30.4)<br>- Cell block (5, 71.4)<br>- Smear (2, 28.6) |
| Diagnosis (n, %) | NSCLC ADC (1, 4.3)<br>NSCLC favor ADC (20, 87.1)<br>NSCLC NOS (1, 4.3)<br>NSCLC ADC + SqCC (1, 4.3) |

ADC: adenocarcinoma; ALK: Anaplastic Lymphoma Receptor Tyrosine Kinase; F: female; KRAS: Kirsten Rat Sarcoma Viral Oncogene Homolog; M: male; MET: MET Proto-Oncogene, Receptor Tyrosine Kinase; n: number; NOS: not otherwise specified; PD-L1: Programmed death-ligand 1; PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; RET: Rearranged During Transfection; ROS1: ROS Proto-Oncogene 1, Receptor Tyrosine Kinase; SqCC: squamous cell carcinoma; y: years.

sis was performed with another narrow NGS panel, namely, SiRe fusion, able to detect *ALK*, *ROS1*, *RET*, *NTRK* gene rearrangements, as well as *MET* exon 14 skipping alterations, as previously described [17,18]. In addition, internal controls were built for the purpose of monitoring the overall quality of the sample and housekeeping genes were used to assess the RNA quality, as previously reported [17,18].

PD-L1 IHC/ICC evaluation was performed by adopting the companion diagnostic kit sp263 assay (Ventana Medical Systems, Oro Valley, AZ, USA), as previously described [10,11]. Briefly, PD-L1 expression was evaluated by using tumor proportion score (TPS), as previously reported [10,11,19].

## Results

### Patient and sample characteristics

We retrospectively retrieved data on a total of 201 samples from advanced stage NSCLC patients who were referred to our laboratory for DNA- and RNA-based NGS analysis as well as PD-L1 expression level evaluation. Overall, 200 (99.5%) were successfully analyzed by our NGS SiRe fusion panel. Of note, 23 (11.5%) SiRe fusion panel positive cases were retrieved. The vast majority of cases were female (17/23, 73.9%), with a median age of 59.0 years (ranging from 33 to 78 years); whereas the remaining cases were male (6/23, 26.1%), with a median age of 54.7 years (ranging from 24 to 79 years). Almost all cases diagnosed with NSCLC were adenocarcinoma (NSCLC favor ADC, 21/23, 91.4%), followed by NSCLC adeno-squamous (1/23, 4.3%) and NSCLC not otherwise specified (NSCLC NOS, 1/23, 4.3%). Considering sample type, the number of histological samples (16, 69.6%) was higher than cytological specimens (7, 30.4%). Histological samples comprised small biopsies (13, 81.3%), and surgical resections (3, 18.7%). As for the cytological samples, they were mostly made up of cell blocks (5, 71.4%), whereas the remaining cases were direct smears (2, 28.6%). Regarding molecular assessment, 11 (5.5%), 2 (1.0%), 9 (4.5%), and 1 (0.5%) out of 200 cases harbored an *ALK*, *ROS1*, *RET* gene rearrangement, or *MET* exon 14 skipping, respectively. Interestingly, only 2 (22.2%) *RET* rearranged cases harbored a concomitant genomic alteration detected at DNA-level (*KRAS* exon 2 p.G12D and *PIK3CA* exon 9 p.E545K). Regarding PD-L1 expression level, about half (11/23, 47.8%) of patients showed PD-L1 expression level between 1-49% (4 *ALK*, and 3 *RET*) or ≥ 50% (2 *ALK*, 1 *ROS1*, and 1 *MET* exon 14 skipping).
Results are summarized in Figures 1-4 and Tables I-II.

### Clinical management

Overall, data on the clinical management of 15 (65.2%) patients were retrieved. Among these, 8 (53.3%), 1 (6.7%), and 6 (40.0%) showed an *ALK*, *ROS1*, or *RET* rearrangement. A concomitant PD-L1 expression 1-49% was observed in 5 instances (33.3%, 4 *ALK*, and 1 *RET*), whereas an expression level ≥ 50% was reported i 2 cases (13.3%, 1 *ALK*, and 1 *ROS1*). In only 1 instance (6.7%) a *RET* rearrangement was associated with a *KRAS* exon 2 p.G12D. Overall, about half of analyzed cases (8, 53.3%) are still undergoing target treatments at the last oncological evaluation (April 18, 2024).
Results are summarized in Table III.

## Discussion

In addition to DNA-based biomarkers, the evaluation of *ALK*, *ROS1*, *RET*, *NTRK* gene rearrangements as well as *MET* exon 14 skipping is crucial for advanced stage NSCLC patients clinical management. In this study, we retrospectively retrieved molecular data of

**Table II.** Clinical and molecular findings of the RNA-based positive population.

| N | Sex | Age | Sample type | Sample subtype | Site | Primitive/ metastasis | Diagnosis | Note | RNA-based alteration | Other alterations/PD-L1 expression level |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | F | 46 | Cytological | Cell block | Lymph node | Metastasis | NSCLC favor ADC | --- | *EML4*(13)::*ALK*(20) | --- |
| 2 | F | 76 | Cytological | Cell block | Lung | Primitive | NSCLC favor ADC | --- | *EML4*(13)::*ALK*(20) | PD-L1 ≥ 50% |
| 3 | F | 69 | Histological | Biopsy | Lung | Primitive | NSCLC favor ADC | --- | *EML4*(6)::*ALK*(20) | PD-L1 ≥ 50% |
| 4 | F | 54 | Histological | Resection | Lung | Primitive | NSCLC ADC | Mucinous, signet ring cells, micropapillary | unknown::*ALK*(20) | PD-L1 1-49% |
| 5 | M | 79 | Histological | Biopsy | Lung | Primitive | NSCLC favor ADC | G2 | unknown::*RET*(12) | *KRAS* p.G12D |
| 6 | F | 78 | Cytological | Smear | Lung | Primitive | NSCLC favor ADC | --- | *KIF5B*(15)::*RET*(12) | *PIK3CA* p.E545K |
| 7 | M | 52 | Histological | Biopsy | Pleura | Metastasis | NSCLC favor ADC | --- | *EML4*(6)::*ALK*(20) | --- |
| 8 | F | 65 | Histological | Biopsy | Lung | Primitive | NSCLC favor ADC | G2, solid | unknown::*ALK*(20) | --- |
| 9 | F | 65 | Cytological | Cell Block | Lung | Primitive | NSCLC favor ADC | G3 | *EML4*(20)::*ALK*(20) | --- |
| 10 | M | 57 | Histological | Biopsy | Brain | Metastasis | NSCLC favor ADC | G3 | unknown::*RET*(12) | PD-L1 1-49% |
| 11 | M | 56 | Cytological | Cell Block | Lung | Primitive | NSCLC favor ADC | --- | unknown::*RET*(12) | --- |
| 12 | F | 60 | Histological | Biopsy | Lung | Primitive | NSCLC favor ADC | Acinar, papillar | *EML4*(13)::*ALK*(20) | PD-L1 1-49% |
| 13 | M | 24 | Histological | Biopsy | Lymph node | Metastasis | NSCLC favor ADC | G3, solid | *CD74*(6)::*ROS1*(34) | PD-L1 ≥50% |
| 14 | F | 72 | Cytological | Cell Block | Lung | Primitive | NSCLC favor ADC | --- | *KIF5B*(15)::*RET*(12) | PD-L1 1-49% |
| 15 | F | 35 | Histological | Biopsy | Breast | Metastasis | NSCLC favor ADC | G2 | *EML4*(13)::*ALK*(20) | PD-L1 1-49% |
| 16 | F | 71 | Histological | Resection | Lymph node | Metastasis | NSCLC favor ADC | G3 | *CCDC6*(1)::*RET*(12) | --- |
| 17 | F | 63 | Histological | Resection | Brain | Metastasis | NSCLC favor ADC | G3 | *KIF5B*(15)::*RET*(12) | --- |
| 18 | F | 47 | Cytological | Smear | Mediastinum | Metastasis | NSCLC ADC + SqCC | --- | *KIF5B*(15)::*RET*(12) | --- |
| 19 | F | 60 | Histological | Biopsy | Liver | Metastasis | NSCLC favor ADC | G3, trabecular, solid | *EML4*(6)::*ALK*(20) | PD-L1 1-49% |
| 20 | F | 37 | Histological | Biopsy | Lymph node | Metastasis | NSCLC favor ADC | Solid | *EML4*(20)::*ALK*(20) | --- |
| 21 | F | 72 | Histological | Biopsy | Lung | Primitive | NSCLC NOS | G3 | *MET* exon 14 skipping | PD-L1 ≥ 50% |
| 22 | F | 33 | Histological | Biopsy | Lung | Primitive | NSCLC favor ADC | --- | *CD74*(6)::*ROS1*(34) | --- |
| 23 | M | 60 | Histological | Biopsy | Lung | Primitive | NSCLC favor ADC | Acinar, papillary, mucinous | *KIF5B*(15)::*RET*(12) | PD-L1 1-49% |

ADC: adenocarcinoma; ALK: Anaplastic Lymphoma Receptor Tyrosine Kinase; CD74: CD74 Molecule, Major Histocompatibility Complex, Class II Invariant Chain; EML4: Echinoderm Microtubule-Associated Protein-Like 4; F: female; KIF5B: Kinesin Family Member 5B; KRAS: Kirsten Rat Sarcoma Viral Oncogene Homolog; M: male; MET: MET Proto-Oncogene, Receptor Tyrosine Kinase; n: number; NOS: not otherwise specified; PD-L1: Programmed death-ligand 1; PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; RET: Rearranged During Transfection; ROS1: ROS Proto-Oncogene 1, Receptor Tyrosine Kinase; SqCC: squamous cell carcinoma.

**Table III.** Clinical management.

| N | Sex | Age | Diagnosis | Note | Fusion/skipping | Other alterations/ PD-L1 expression level | First Line treatment | First Line treatment Starting Date | First Line treatment End Date | Second Line treatment | Second Line treatment Starting Date | Second Line treatment End Date | Third Line treatment | Third Line treatment Starting Date | Third Line treatment End Date |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 | F | 69 | NSCLC favor ADC | --- | *EML4*(6)::*ALK*(20) | PD-L1 ≥ 50% | Brigatinib | December 2020 | July 2021 | NA | NA | NA | NA | NA | NA |
| 4 | F | 54 | NSCLC ADC | Mucinous, signet ring cells, micropapillar | Unknown::*ALK*(20) | PD-L1 1-49% | Alectinib | February 2021 | Ongoing | NA | NA | NA | NA | NA | NA |
| 5 | M | 79 | NSCLC favor ADC | G2 | Unknown::*RET*(12) | *KRAS* p.G12D | Brain RT | December 2021 | May 2022 | NA | NA | NA | NA | NA | NA |
| 8 | F | 65 | NSCLC favor ADC | G2, solid | Unknown::*ALK*(20) | --- | Alectinib | September 2021 | Ongoing | NA | NA | NA | NA | NA | NA |
| 9 | F | 65 | NSCLC favor ADC | G3 | *EML4*(20)::*ALK*(20) | --- | Alectinib | February 2021 | Ongoing | NA | NA | NA | NA | NA | NA |
| 10 | M | 57 | NSCLC favor ADC | G3 | Unknown::*RET*(12) | PD-L1 1-49% | Carboplatin + pemetrexed | November 2021 | January 2022 | NA | NA | NA | NA | NA | NA |
| 11 | M | 56 | NSCLC favor ADC | --- | Unknown::*RET*(12) | --- | Carboplatin + paclitaxel + RT | October 2021 | September 2022 | Selpercatinib | September 2022 | April 2023 | Atezolizumab | April 2023 | Ongoing |
| 12 | F | 60 | NSCLC favor ADC | Acinar, papillar | *EML4*(13)::*ALK*(20) | PD-L1 1-49% | Alectinib | November 2021 | Ongoing | NA | NA | NA | NA | NA | NA |
| 13 | M | 24 | NSCLC favor ADC | G3, solid | *CD74*(6)::*ROS1*(34) | PD-L1 ≥ 50% | Entrectinib | March 2022 | March 2023 | Best supportive care | March 2023 | Ongoing | NA | NA | NA |
| 15 | F | 35 | NSCLC favor ADC | G2 | *EML4*(13)::*ALK*(20) | PD-L1 1-49% | Alectinib | February 2022 | Ongoing | NA | NA | NA | NA | NA | NA |
| 16 | F | 71 | NSCLC favor ADC | G3 | *CCDC6*(1)::*RET*(12) | --- | Carboplatin + pemetrexed, then pemetrexed | April 2019 | January 2022 | Selpercatinib | January 2022 | Ongoing | NA | NA | NA |
| 17 | F | 63 | NSCLC favor ADC | G3 | *KIF5B*(15)::*RET*(12) | --- | Selpercatinib | October 2022 | Ongoing | NA | NA | NA | NA | NA | NA |
| 18 | F | 47 | NSCLC ADC + SqCC | --- | *KIF5B*(15)::*RET*(12) | --- | Carboplatin + paclitaxel + pembrolizumab, then pembrolizumab | May 2022 | Ongoing | NA | NA | NA | NA | NA | NA |
| 19 | F | 60 | NSCLC favor ADC | G3, trabecular, solid | *EML4*(6)::*ALK*(20) | PD-L1 1-49% | Alectinib | May 2022 | July 2022 | NA | NA | NA | NA | NA | NA |
| 20* | F | 37 | NSCLC favor ADC | Solid | *EML4*(20)::*ALK*(20) | --- | Alectinib | May 2020 | July 2022 | Lorlatinib | July 2022 | November 2022 | Brigatinib | November 2022 | March 2023 |

ADC: adenocarcinoma; ALK: Anaplastic Lymphoma Receptor Tyrosine Kinase; CD74: CD74 Molecule, Major Histocompatibility Complex, Class II Invariant Chain; EML4: Echinoderm Microtubule-Associated Protein-Like 4; F: female; KIF5B: Kinesin Family Member 5B; KRAS: Kirsten Rat Sarcoma Viral Oncogene Homolog; M: male; n: number; NA: not assessed; NOS: not otherwise specified; PD-L1: Programmed death-ligand 1; RET: Rearranged During Transfection; ROS1: ROS Proto-Oncogene 1, Receptor Tyrosine Kinase; RT: radiotherapy; SqCC: squamous cell carcinoma.

* Patient 20: Fourth Line treatment: Carboplatin + pemetrexed, then pemetrexed; Fourth Line treatment Starting Date: March 2023; Fourth Line treatment End Date: August 2023; Fifth Line treatment: Docetaxel; Fifth Line treatment Starting Date: August 2023; Fifth Line treatment End Date: January 2024; Sixth Line treatment: Nivolumab; Sixth Line treatment Starting Date: January 2024; Sixth Line treatment End Date: Ongoing.

a total of 201 advanced stage NSCLC patients who were referred to our referral laboratory for the molecular evaluation of RNA-based biomarkers. Focusing the attention on the 23 positive cases in our series, the vast majority (17/23, 73.9%) of analyzed samples were represented by small tissue specimens. Overall, we confirmed the feasibility of a complementary DNA- and RNA-based NGS approach with narrow, custom, gene panels, namely SiRe® *and* SiRe fusion, to optimize small tissue samples for molecular analysis respect to the adoption of single gene assays [20-23]. In our study, 11 (5.5%), 2 (1.0%), 9 (4.5%), and 1 (0.5%) out of 200 successfully analyzed cases harbored an *ALK*, *ROS1*, *RET* gene rearrangement, or *MET* exon 14 skipping, respectively. Similar with those reported in the literature, almost all cases were diagnosed with NSCLC were ADC (21/23, 91.4%) [24]. As expected, *ALK* rearranged cases were most frequent in young (< 65 years, 7/11, 63.6%), female (10/11, 90.9%) ADC patients (11/11, 100.0%).[23] In almost all instances (9/11, 81.8%) the fusion partner was Echinoderm Microtubule-Associated Protein-Like 4 (*EML4*) gene [24]. Similarly, the only 2 CD74 Molecule, Major Histocompatibility Complex, Class II Invariant Chain (*CD74*)::*ROS1* rearranged patients were young patients (22 and 33 years, respectively) with an ADC morphology [24]. Considering *RET* rearranged cases, no significant differences were reported regarding sex (5 female and 4 male) or age (5 < 65 years and 4 ≥ 65 years) [24]. Overall, almost all cases (8/9, 88.9%) were diagnosed as NSCLC favor ADC [24]. As reported in the literature, the most common fusion partner was Kinesin Family Member 5B (*KIF5B*) gene [24]. Noteworthy, in 2 instances *RET* rearrangements were identified in association with a concomitant DNA-based alteration (1 *KRAS* exon 2 p.G12D and 1 *PIK3CA* exon 9 p.E545K). In addition, only 1 (0.5%) *MET* exon 14 skipping was detected. Interestingly, in this case a PD-L1 expression ≥ 50% was observed. As reported in the IMMUNOTARGET registry study, patients harboring a concomitant *MET* exon 14 skipping and PD-L1 expression ≥ 50% may be sensitive to ICIs; in fact, 23.4% of patients with *MET* alterations were long-term responders to ICIs, second only to *KRAS* mutated NSCLC [25]. Regarding PD-L1 expression, in our study other 10 rearranged cases showed a concomitant PD-L1 ≥ 1% ( 6 *ALK*, 1 *ROS1*, and 3 *RET*). However, taking into account the results of IMMUNOTARGET registry study, ICIs monotherapy is not recommended in these patients [25].

As for the data on treatment regimens, 8 (53.3%) patients (#4, #8, #9, #12, #15, #16, #17, #19, Table III) are still in treatment at the last oncological evaluation (April 18, 2024) with a specific target treatment for the gene rearrangement identified by our SiRe fusion panel. Among these, clinical efficacy of specific target treatments was observed in 2 *ALK* rearranged patients in whom gene rearrangements were identified without the knowledge of the specific fusion partner (patients #4 and #8 Table III). In these cases, despite Ambrosini-Spaltro et al. highlighted that patients with gene fusions with unknown partners showed a poor response to targeted therapy [26], our data, albeit limited, may suggest to further investigate the role of gene fusions with unknown partners in clinical trials for target treatment administration. Considering single gene testing approaches, fluorescence in situ hybridization (FISH) is still considered the "gold standard" methodology for gene rearrangement detection and does not require a previous knowledge of the fusion partner. Nevertheless, acting at the DNA level, FISH suffers from "false positive" results (not all the detected DNA rearrangements determine an expressed fusion transcript). In addition, break apart probes can miss small intrachromosomal rearrangements. Of note, FISH is time consuming, influenced by interobserver variability and has a limited multiplexing power [27]. IHC/ICC has the advantage to be more familiar to all anatomic pathologists, as well as less time consuming, automater, less costly, and different clinically validated antibodies are commercially available. However, it can be influenced by interobserver variability, has a limited multiplexing power, and, except for ALK protein evaluation, requires confirmation by orthogonal techniques [27]. Finally, retro-transcriptase polymerase chain reaction (RT-PCR), despite the high sensitivity for fusion transcripts at RNA levels, is able to identify only known gene fusions, missing all the unknown variants [26]. Thus, in this complex scenario, as RNA-based NGS approach, through its multiplexing power and the possibility to identify known and unknown variants, represents a valid solution to overcome all these limitations [27]. However, all that glitters is not gold. RNA-based NGS analysis can be hampered by low RNA quality and purity. Moreover, since RNA is less stable than DNA, special care must be taken during the pre-analytical phase to minimize the risk of false-negative results. In addition, RNA-based NGS-based analyses also requires trained personnel and good communication must be established with clinicians to ensure the correct interpretation of NGS reports [27]. Other limitations are specifically related to the use of a narrow panel instead of comprehensive genome profile or whole exome sequencing approaches, including the risk of overlooking potentially actionable translocations, such as those involving *NTRK2* and *-3*, as well as neuregulin 1 (*NRG1*). This could possibly prevent some patients from receiving effective treatment, also

considering that the list of actionable alterations is constantly expanding, with several novel agents currently being evaluated in clinical trials (e.g., seribantumab in tumors with *NRG1* fusions) [28].

In conclusion, in this study we provide a real world experience on RNA-based NGS analysis in patients with advanced stage NSCLC. The most significant limitations of our study were the limited number of cases and the lack of clinical data in some instances. Further studies are required to better assess the role of the complex genomic landscape in advanced stage NSCLC patients, not only on tissue but also on liquid biopsy specimens [29].

### Conflict of interest statement

Umberto Malapelle has received personal fees (as consultant and/or speaker bureau) from Boehringer Ingelheim, Roche, MSD, Amgen, Thermo Fisher Scientific, Eli Lilly, Diaceutics, GSK, Merck and AstraZeneca, Janssen, Diatech, Novartis and Hedera for work performed unrelated to the current work. Giancarlo Troncone reports personal fees (as speaker bureau or advisor) from Roche, MSD, Pfizer and Bayer, unrelated to the current work.

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### Authors contribution

Conceptualization, Pasquale Pisapia, Umberto 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, Pasquale Pisapia; Writing – Review & Editing, all authors; Visualization, all authors; Supervision, Pasquale Pisapia, Giancarlo Troncone, Umberto Malapelle; Project Administration, Pasquale Pisapia, Giancarlo Troncone, Umberto Malapelle; Funding acquisition, Giancarlo Troncone.

### Ethical consideration

Institutional Review Board approval was waived since this study was retrospective and, thus, required no deviation from the standard of care. Written informed consent was obtained from all the patients in accordance with the general authorization for processing personal data for scientific research purposes from "The Italian Data Protection Authority" (http://www.garanteprivacy.it/web/guest/home/docweb/-/docweb-display/export/2485392). All information regarding human material was managed using anonymous numerical codes, and all samples were handled in compliance with the Declaration of Helsinki (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/).

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