# UNIVERSITA' DEGLI STUDI DI NAPOLI FEDERICO II

## DIPARTIMENTO DI SANITA' PUBBLICA

### DOTTORATO DI RICERCA IN SANITA' PUBBLICA E MEDICINA PREVENTIVA - XXXII CICLO

# *Development of an integrable RNA based Gene Panel for Next-Generation Sequencing of Clinically Relevant Gene Fusions in Non-Small Cell Lung Cancer Patients*


**Tutor:**

Ch.mo Prof. Giancarlo Troncone

**Candidato:**

Dott. Riccardo Smeraglio

**Coordinatore:**

Ch.mo Prof. Giancarlo Troncone


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**1 - Abstract**

**Introduction**: Non-small cell lung cancer (NSCLC) still represents the main cause of cancer mortality worldwide. However, targeted treatments have significantly improved the clinical outcome of those patients with non-squamous NSCLC harboring not only point mutations and indertions/deletions (indels), but also gene rearrangements. Currently, immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) are considered the "gold standard" methodologies for the identification of these genomic alterations but feature some technical and interpretative limitations. Therefore, the aim of this study is to validate a novel narrow custom next generation sequencing gene panel, termed SiRe gene fusion panel, able to detect gene rearrangements and other aberrations (such *MET* exon 14 skipping) for the administration of specific tyrosine kinase inhibitors.

**Material and methods**: firstly, we assessed the minimal complementary DNA (cDNA) input and the limit of detection (LoD) in different cell lines and standardized reference samples built adopting different fixation modalities. Then, we retrospectively selected 48 previously genotyped archival lung adenocarcinoma histological and cytological (cell blocks) samples to evaluate the feasibility of our panel in the routine clinical setting.

**Results**: SiRe fusion panel was able to detect all fusions and a splicing event harbored in a RNA pool diluted up to 2 ng/µL. It also successfully analyzed 46 (95.8%) out of 48 routine clinical samples. Among these, 43 (93.5%) out of 46 samples showed a concordand result with conventional techniques.

**Conclusions:** SiRe fusion panel represents a valid and robust diagnostic tool for the detection of clinically relevant gene fusions and splicing events in advanced stage NSCLC patients.

## *2 – Introduction*

Lung cancer, and in particular non-small cell lung cancer (NSCLC), is the principal cause of cancer mortality worldwide.[1] In fact, lung cancer is a multifactorial disease commonly diagnosed in the advanced stages (IIIB- IV). In the latest stages, treatment options are generally limited to the conventional chemotherapy, but clinical outcome is very poor. To date, targeted treatments represents important "arrows in the quiver" for clinical oncologists in particular for those non-squamous NSCLC patients harboring point mutations and insertions/deletions (indels) but also gene rearrangements.[2-9] In the recent years, the National Comprehensive Cancer Network (NCCN), the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC) and the Association for Molecular Pathology (AMP) established a panel of "must test genes" that includes gene fusions targetable by specific tyrosine-kinase inhibitors (TKIs).[10, 11] Anaplastic Lymphoma Kinase (*ALK*) gene fusions, occurring in about 3–6% of NSCLC patients, are predictive of responsiveness to crizotinib, alectinib, and brigatinib treatments.[12-17] Crizotinib is also effective in NSCLC patinets harboring ROS Proto-Oncogene 1 Receptor Tyrosine Kinase (*ROS1*) gene fusions, identified in about 1–2% of patients.[18-20] Despite the rarity, Neurotrophic Receptor Tyrosine Kinase (*NTRK*) (<1%) gene fusions acquired a relevant role in NSCLC patients due to the responsiveness to larotrecnib and entrectinib.[21-25] Finally, REarranged during Transfection (*RET*) gene fusions (1–2% of NSCLC patients) showed a high sensitivity to selpercatinib and pralsetinib.[26-28] To date, immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) represent the "gold standard" methodologies for the identification of gene rearrangements but feature some technical and interpretative limitations, in particular high time consuming, costs and, in some cases, optimization of pre analytical variables are essential to generate an interpretable result.[29-32] In addition, another important limitations related to increasing number of clinical relevant biomarkers is associated with the limited amount of available tissue samples in advanced stage NSCLC patients for morph-molecular purposes.[32] Next generation sequencing (NGS) represents a valid tool able to overcome the limitations of

"single gene" testing assays. In particular, NGS technology is able to cover not only DNA-based biomarkers but also RNA-based, including gene fusions and other aberrations of clinical relevance such as the MET Proto-Oncogene, Receptor Tyrosine Kinase (*MET*) exon 14 skipping.[33-35] Several NGS panels able to identify chromosomal rearrangements are commercially available.[33-35] Nevertheless, these commercially available NGS panels cover not only pulmonary clinically relevant biomarkers but a large number of biomarkers involved in several tumor types. Thus, there is an unmet need related to the development of custom NGS panel tailored on the detection of DNA and RNA alterations for lung cancer patients. Our predictive molecular laboratory at the Department of Public Health of the University of Naples Federico II, have recently developed, validated and introduced in the diagnostic routine practice a custom DNA-based NGS panel able to simultaneously cover hotspot mutations of clinical interest for NSCLC patients.[36, 37] In addition, in order to cover all clinical relevant biomarkers of NSCLC patients, we designed a narrow RNA based NGS panel, termed SiRe fusion panel, able to cover all the clinically relevant gene fusions and splicing events in NSCLC patients starting from a low amount of neoplastic cells.

Therefore, the aim of this project is to evaluate the analytical and clinical performance of this narrow RNA panel for the analysis of clinically relevant fusions in *ALK*, *ROS1*, *RET* and *NTRK* genes and *MET* exon 14 skipping.

## *3 - Material and Methods*

### *3.1 - Panel Design*

The SiRe fusion primers pool was developed by using AmpliSeq designer software v.7.4 to cover the most clinically relevant rearrangements in four genes (*ALK, ROS1, RET, NTRK,*) and *MET* exon 14 skipping in NSCLC patients. A single primer pool leading to the selection of 91 amplicons (ranging from 125 to175 bp) enabled us to cover selected genomic alterations in the target genes. The panel was designed to analyse complementary DNA (cDNA) synthesized after RNA retro-

transcription purified from several sample preparations of advanced stage cancers patients, including formalin fixed and paraffin embedded (FFPE) sections, cytopathological specimens and body fluid.

### 3.2 - Analytical Controls

The evaluation of analytical performance of the SiRe RNA fusion panel, in terms of minimal cDNA input required and limit of detection (LoD), was carried out by analyzing molecular reference standards. First of all, cell lines, purchased from the American Type Culture Collection and cultured in RPMI medium 10% fetal bovine serum under standard conditions, were used to assess and to confirm the reference range of SiRe fusion primers pool. As reported in Table 1, cell lines harbored different clinically relevant gene fusions and *MET* exon 14 skipping alteration. Nucleic acids extracted from the expanded cell lines were also used to create reference standard for NGS analysis.

**Table 1**. Cell lines harboring clinically relevant gene fusions for NSCLC patients

| Cell line | Mutation |
|---|---|
| H3122 | *EML4*(13)-*ALK*(20) |
| H2228 | *EML4*(6)-*ALK*(20) |
| Hs746T | *MET* exon 14 skipping |
| H596 | *MET* exon 14 skipping |
| HCC-78 | *SLC34A2*(4)-*ROS1*(32) |
| LC2-ad | *CCDC6*(1)-*RET*(12) |
| EBC-1 | *MET* Amplification |
| SUDHL-1 | *NPM1-ALK* |
| NTRK1cl | *TPM3*(8)-*NTRK1*(10) |

Abbreviations: Abbreviations: *ALK*: Anaplastic Lymphoma Kinase; *CCDC6*: Coiled-Coil Domain Containing 6; chr: chromosome; *EML4*: Echinoderm Microtubule-Associated Protein-Like 4; *MET*: MET Proto-Oncogene, Receptor Tyrosine Kinase; NGS: next generation sequencing; *NPM1*: Nucleophosmin 1; *NTRK1*: Neurotrophic Receptor Tyrosine Kinase 1; *RET*: Rearranged During Transfection; *ROS1*: ROS Proto-Oncogene 1, Receptor Tyrosine Kinase; *SLC34A2*: Solute Carrier Family 34 Member 2; *TPM3*: Tropomyosin 3.

In addition, a set of reference standard slides containing cell lines engineered to harbor Solute Carrier Family 34 Member 2 [*SLC34A2*](4)-*ROS1*(32), Coiled-Coil Domain Containing 6 [*CCDC6*](1)-*RET*(12), Echinoderm Microtubule-Associated Protein-Like 4 [*EML4*](10)-*ALK*(20), and Tropomyosin 3 [*TPM3*](8)-*NTRK1*(10) [Table 2] (purchased from Horizon Diagnostics HDx Cambridge, United Kingdom) were evaluated in order to verify if different fixation modalities (methanol or ethanol) may interfere with the molecular analysis. In details, engineered cells built to harbor all the clinically relevant fusion genes covered by SiRe fusion panel were fixed with 100% ethanol at -20°C for 10 minutes or 70% methanol at room temperature for four hours. A number of 100000 fixed cells were pipetted onto each slide and allowed to dry. Prepared slides were stored at -20°C overnight. A set of five methanol and ethanol fixed slides were produced for mutant and wild type controls, respectively.

**Table 2.** Standard references with the corresponding fixation modalities.

| Item number | Description | Number of slides | Internal reference |
|---|---|---|---|
| HD – D187 | Cytology Fusion Negative ETOH fixed RNA | 5 | FN-ETOH |
| HD – D188 | Cytology Fusion Positive ETOH fixed RNA | 5 | FP-ETOH |
| HD – D197 | Cytology Fusion Negative MEOH fixed RNA | 5 | FN-MEOH |
| HD – D198 | Cytology Fusion Positive MEOH fixed RNA | 5 | FP-MEOH |

### 3.3 - Routine diagnostic samples

Finally, in order to evaluate SiRe fusion performance on diagnostic routine samples, a series of n = 48 archival diagnostic tissue specimens, including FFPE histological or cell block specimens, from NSCLC patients (25 men and 23 women; mean age: 56.0 years) previously tested by using IHC, FISH or NGS based approaches and harbouring *ALK*, *ROS1*, *RET* gene fusions or *MET* exon 14 skipping were selected from different Italian institutions participating in the validation study (University of Naples Federico II, Naples, Italy; A.O.R.N. Antonio Cardarelli, Naples, Italy; San Luigi University Hospital, University of Turin, Orbassano, Italy; San Gerardo Hospital, University of Milano-Bicocca, Monza, Italy).

### 3.4 - RNA extraction, retrotrascription and quantification

Regarding molecular analysis, RNA from reference standard was extracted by using All Prep DNA - RNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer instructions and re-suspending in 50 µl of RNAsi/DNAsi free water (Ambion, Thermo Fisher Scientific, Waltham, MA). RNA was directly purified from cell lines by using the High Pure RNA Isolation kit (Roche Diagnostics, Basel, Switzerland) in Pangea Biotech laboratory (Barcelona, Spain), following manufacturer instructions. TapeStation 4200 system (Agilent Technologies, Santa Clara, CA) was adopted to evaluate RNA concentration (ng/µl) and estimate RNA quality by inspecting RNA integrity number (RIN). The retro - transcription step was carried out by using SuperScript IV VILO Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. Finally, cDNA quantification was realized on TapeStation 4200 by using D1000 Genomic assay (Agilent Technologies). Discordant results were confirmed with a full-closed real time polymerase chain reaction (RT-PCR) approach (Easy PGX platform, Diatech Pharmacogenetics, Jesi, Italy).

### 3.5 - Libraries preparation and NGS analysis with SiRe RNA fusion panel

The SiRe® RNA fusion primers pool was designed to reveal the most clinically relevant

translocations in *ALK, ROS1, RET, NTRK* and *MET* exon 14 skipping alterations. Libraries were constructed and purified on the Ion Chef instrument (Thermo Fisher Scientific) following the manufacturer procedures. Library generation was as follows: 6 microliters of cDNA (with an optimal concentration of 2 ng/µl) were dispensed on Ion Code plates and amplified using Ion AmpliSeq DL8 (Thermo Fisher Scientific). We used 24 cycles for cfDNA amplification and 7 cycles for library re-amplification after barcoding, under the thermal conditions defined by the manufacturer protocol. Purified libraries were diluted to 60pM and pooled together. The pooled libraries were re-loaded into the Ion Chef instrument, and templates were prepared using the S5 510-520-530 chef Kit (Thermo Fisher Scientific). Finally, templates were loaded into the 520 chip and sequenced on S5 NGS platform (Thermo Fisher Scientific). In each run, n=16 purified libraries were simultaneously analysed. The results interpretation was carried out by using a proprietary pipeline developed by the Department of Public Health on IonReporter Software (Thermo Fisher Scientific).

## 4 – Results

### 4.1 Panel design

Overall, 91 primer pairs (one pool) were able to cover the 100% of gene fusions reported in the material and methods section, reaching a 5000X of coverage for each target. The obtained fusion primer pool was fully compatible with our previously validated SiRe DNA panel due to the fact that both work under the same thermal condition. This aspect leads to the integration of RNA and DNA analysis for the same sample set in a single NGS run.

### 4.2 - Cell lines input quantity optimization and Limit of Detection

Overall, all standard references were successfully analyzed (100.0%). RNA concentration showed a median value of 15.3 ng/µL, (ranging from 2.12 ng/µL for NTRK1cl to 31.4 ng/µL for H596).

Moreover, RNA integrity number (RIN) median value was 2.9 (ranging from 1.9 for LC2-ad to 5.5 for H596) (Table 3).

**Table 3.** RNA quantification and quantification data performed on cell lines by using TapeStation 4200.

| **Sample** | **Concentration (ng/µL)** | **RNA Integrity Number (RIN)** |
|---|---|---|
| H3122 | 20.9 | 3.3 |
| H2228 | 15.2 | 2.9 |
| Hs746T | 28.3 | 2.3 |
| H596 | 31.4 | 5.5 |
| HCC78 | 3.4 | 4.2 |
| LC2-ad | 8.4 | 1.9 |
| EBC1 | 23.2 | 2.9 |
| SUDHL-1 | 4.6 | 1.6 |
| NTRK1cl | 2.1 | 2.0 |

### 4.3 - RNA evaluation from standard references

RNA isolated from all the mutant cell lines were mixed to generate a set of five different serial diluitions points (20 ng/µl, 10 ng/µl, 2 ng/µl, 0.5 ng/µl, 0.1 ng/µl). The pooled RNA was analyzed as previously reported. Interestingly, a median value of 73.97% (ranging from 64.51% to 85.60%), of 93.00 bp (ranging from 48.00 to 117.00), of 21427.80 (ranging from 13,642.00 to 27,230.00) and of 20432.40 (ranging from 28,229.00 to 13,784.00) for target reads, reads lenght, mapped reads and coverage uniformity, were reached, respectively. SiRe RNA fusion panel was able to detect all fusions and the splicing event harbored by RNA pool until the 2 ng/µl dilution (Table 4).

**Table 4**. Analytical evaluation of starting amount required to identify fusion events in cell lines by using SiRe RNA panel.

| ng/µl (cell line mix) | Filter | Fusion Detected | Splicing Detected |
|---|---|---|---|
| 20.0 | PASS | 6/6 | 1/1 |
| 10.0 | PASS | 6/6 | 1/1 |
| 2.0 | PASS | 6/6 | 1/1 |
| 0.5 | PASS | n.a. | n.a. |
| 0.1 | PASS | n.a. | n.a |

### 4.4 - Reference Range and Fixation modalities

All processed standard references samples passed the quality filters. NGS run parameters in mutant cell lines were evaluated in order to assess the analytical performance of the SiRe RNA fusion panel to detect clinically relevant rearrangements. Regarding run parameters, an average of 99.70% (ranging from 99.13% to 99.98%) reads on target was reported. The median read length was of 103.55 bp (ranging from 93.00 to 114.00). Concerning the number of mapped reads, an average of 311798.44 (ranging from 60530.00 to 948223.00) was reached. Regarding the uniformity of coverage, an average of 318415.89 (ranging from 60562.00 to 96378.00) was obatined. Interestingly, all the gene fusions as well as *MET* exon 14 skipping were detected by using SiRe fusion panel (Tables 5-8). Intriguingly, all gene alterations were successfully detected when diluting the four different cell lines at 50%, 25%, and 10%. Interestingly, better results were obtained in methanol fixed respect to ethanol fixed group.

**Table 5.** FP ME-OH standard references analysis by using SiRe RNA panel.

| Locus | Type | Filter | Genes (exons) | Read counts | Detection |
|---|---|---|---|---|---|
| chr6:170871321 | EXPR_CON- | PASS | *TBP* | 52448 | Present |
| chr12:53585787 | EXPR_CON- | PASS | *ITGB7* | 10874 | Present |
| chr8:128751265 | EXPR_CON- | PASS | *MYC* | 163240 | Present |
| chr11:118960975 | EXPR_CON- | PASS | *HMBS* | 87238 | Present |
| chr1:156104319 | EXPR_CON- | PASS | *LMNA* | 174150 | Present |
| chr4:25665952 – chr6:117650609 | FUSION | PASS | *SLC34A2*(4) - *ROS1*(32) | 33224 | Present |
| chr10:61665880 – chr10:43612032 | FUSION | PASS | *CCDC6*(1) - *RET*(12) | 31148 | Present |

| | | | | | |
|---|---|---|---|---|---|
| chr2:42522656 – chr2:29446394 | FUSION | PASS | *EML4*(10) - *ALK*(20) | 61 | Present |
| chr4:25665952 – chr6:117645578 | FUSION | PASS | *SLC34A2*(4) - *ROS1*(34) | 402 | Present |
| chr1:154142878 – chr1:156844363 | FUSION | PASS | *TPM3*(8) - *NTRK1*(10) | 1161 | Present-Non-Targeted |

Abbreviations: *ALK*: Anaplastic Lymphoma Kinase; *CCDC6*: Coiled-Coil Domain Containing 6; chr: chromosome; *EML4*: Echinoderm Microtubule-Associated Protein-Like 4; FP: fusion positive; *HMBS*: Hydroxymethylbilane Synthase; *ITGB7*: Integrin Subunit Beta 7; *LMNA*: Lamin A/C; MEOH: methanol; *NTRK1*: Neurotrophic Receptor Tyrosine Kinase 1; *TBP*: TATA-Box Binding Protein; *MYC*: MYC Proto-Oncogene, BHLH Transcription Factor; *RET*: Rearranged During Transfection; *ROS1*: ROS Proto-Oncogene 1, Receptor Tyrosine Kinase; *SLC34A2*: Solute Carrier Family 34 Member 2; *TPM3*: Tropomyosin 3.

**Table 6**. FP Et-OH standard references analysis by using SiRe RNA panel.

| Locus | Type | Filter | Genes (exons) | Read counts | Detection |
|---|---|---|---|---|---|
| chr6:170871321 | EXPR_CON- | PASS | *TBP* | 63629 | Present |
| chr12:53585787 | EXPR_CON- | PASS | *ITGB7* | 4434 | Present |
| chr8:128751265 | EXPR_CON- | PASS | *MYC* | 108560 | Present |
| chr11:118960975 | EXPR_CON- | PASS | *HMBS* | 68122 | Present |
| chr1:156104319 | EXPR_CON- | PASS | *LMNA* | 131058 | Present |
| chr4:25665952 – chr6:117650609 | FUSION | PASS | *SLC34A2*(4) - *ROS1*(32) | 46965 | Present |
| chr10:61665880 – chr10:43612032 | FUSION | PASS | *CCDC6*(1) - *RET*(12) | 33085 | Present |

| | | | | | |
|---|---|---|---|---|---|
| chr2:42522656 – chr2:29446394 | FUSION | PASS | *EML4*(10) - *ALK*(20) | 52 | Present |
| chr4:25665952 – chr6:117645578 | FUSION | PASS | *SLC34A2*(4) - *ROS1*(34) | 910 | Present |
| chr1:154142878 – chr1:156844363 | FUSION | PASS | *TPM3*(8) - *NTRK1*(10) | 1591 | Present-Non-Targeted |

Abbreviations: *ALK*: Anaplastic Lymphoma Kinase; *CCDC6*: Coiled-Coil Domain Containing 6; chr: chromosome; *EML4*: Echinoderm Microtubule-Associated Protein-Like 4; ETOH: ethanol; FP: fusion positive; *HMBS*: Hydroxymethylbilane Synthase; ITGB7: Integrin Subunit Beta 7; LMNA: Lamin A/C; *NTRK1*: Neurotrophic Receptor Tyrosine Kinase 1; *TBP*: TATA-Box Binding Protein; *MYC*: MYC Proto-Oncogene, BHLH Transcription Factor; *RET*: Rearranged During Transfection; *ROS1*: ROS Proto-Oncogene 1, Receptor Tyrosine Kinase; *SLC34A2*: Solute Carrier Family 34 Member 2; *TPM3*: Tropomyosin 3.

**Table 7.** FN ME-OH standard references analysis by using SiRe RNA panel.

| Locus | Type | Filter | Genes (exons) | Read counts | Detection |
|---|---|---|---|---|---|
| chr6:170871321 | EXPR_CON- | PASS | *TBP* | 51348 | Present |
| chr12:53585787 | EXPR_CON- | PASS | *ITGB7* | 11732 | Present |
| chr8:128751265 | EXPR_CON- | PASS | *MYC* | 155179 | Present |
| chr11:118960975 | EXPR_CON- | PASS | *HMBS* | 86799 | Present |
| chr1:156104319 | EXPR_CON- | PASS | *LMNA* | 176573 | Present |

Abbreviations: chr: chromosome; *EML4*: Echinoderm Microtubule-Associated Protein-Like 4; FN: fusion negative; *HMBS*: Hydroxymethylbilane Synthase; *ITGB7*: Integrin Subunit Beta 7; *LMNA*: Lamin A/C; MEOH: methanol; *TBP*: TATA-Box Binding Protein; *MYC*: MYC Proto-Oncogene, BHLH Transcription Factor.

**Table 8.** FN Et-OH standard references analysis by using SiRe RNA panel.

| Locus | Type | Filter | Genes (exons) | Read counts | Detection |
|---|---|---|---|---|---|
| chr6:170871321 | EXPR_CON- | PASS | *TBP* | 64528 | Present |
| chr12:53585787 | EXPR_CON- | PASS | *ITGB7* | 4321 | Present |
| chr8:128751265 | EXPR_CON- | PASS | *MYC* | 107905 | Present |
| chr11:118960975 | EXPR_CON- | PASS | *HMBS* | 66435 | Present |
| chr1:156104319 | EXPR_CON- | PASS | *LMNA* | 130763 | Present |

Abbreviations: chr: chromosome; *EML4*: Echinoderm Microtubule-Associated Protein-Like 4; ETOH: ethanol; FN: fusion negative; *HMBS*: Hydroxymethylbilane Synthase; *ITGB7*: Integrin Subunit Beta 7; *LMNA*: Lamin A/C; *TBP*: TATA-Box Binding Protein; *MYC*: MYC Proto-Oncogene, BHLH Transcription Factor.

All mutations were further confirmed on residual extracted RNA from fusion positive methanol fixed samples by using *ALK*, *ROS1*, *RET* fusions and *MET* exon 14 skipping kit and *NTRK* fusion kit by using Easy PGX platform (Table 9).

**Table 9**. FP ME-OH standard references analysis by using Easy PGX system.

| | ***ALK*** | ***ROS1* ex 32** | ***ROS1* ex34** | ***ROS1* ex 35-36** | ***RET* ex12** | ***RET* ex 8-11** | ***MET* ex 14 skipping** | ***NTRK1* ex 9-10** | ***NTRK1* ex 10** | ***NTRK1* ex 11-12del** | ***NTRK1* ex 12** | ***NTRK2* ex 12-15** | ***NTRK2* ex 16-17** | ***NTRK3* ex 14** | ***NTRK3* ex 15** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FP - MEOH | MUT | MUT | MUT | WT | MUT | WT | WT | WT | WT | WT | WT | WT | WT | WT | MUT |

Abbreviations: *ALK*: Anaplastic Lymphoma Kinase; ex: exon; FP: fusion positive; MEOH: methanol; *MET*: MET Proto-Oncogene, Receptor Tyrosine Kinase; *NTRK*: Neurotrophic Receptor Tyrosine Kinase; *RET*: Rearranged During Transfection; *ROS1*: ROS Proto-Oncogene 1, Receptor Tyrosine Kinase.

### 4.5 - Validation of SiRe fusion panel on routine samples

Overall, 48 FFPE previously tested samples from advanced stage NSCLC patients were analysed. Among them, 19 (39.6%) out of 48 and 29 (60.4%) out of 48 were cytological or histological samples. Overall, 46 out of 48 (95.8%) samples were successfully analyzed by adopting SiRe fusion gene panel. A perfect concordance with the original methodology was highlighted in 43 (93.5%) out of 46 analyzed cases. Only 3 (6.5%) out of 46 instances did not show a concordant result with conventional technology. (Table 10) All three discordant samples were further analyzed by using an automated RT-PCR approach (Easy PGX platform, Diatech Pharmacogenetics) for *ALK*, *ROS1*, *RET* fusions and *MET* exon 14 skipping, and *NTRK* fusion kits. In all instances (3/3, 100.0%) the obtained results were concordant with SiRe fusion panel results.

**Table 10**. Molecular results of fusion events and Met exon 14 skipping alteration by adopting SiRe RNA panel on a retrospective cohort of NSCLC patients.

| N | Locus | Genes (Exon) | Reads count | Original detection method |
|---|---|---|---|---|
| 1 | chr2:42491871-chr2:29446394 | *EML4*(6)-*ALK*(20) | 5321 | IHC |
| 2 | chr2:42492091-chr2:29446394 | *EML4*(6)-*ALK*(20) | 6285 | IHC |
| 3 | chr2:42491871-chr2:29446394 | *EML4*(6)-*ALK*(20) | 1662 | IHC |
| 4 | chr2:42522656-chr2:29446394 | *EML4*(13)-*ALK*(20) | 2391 | IHC |
| 5 | chr20:43959006-chr6:117645578 | *SDC4*(4)-*ROS1*(34) | 94 | IHC |
| 6 | chr5:149784243-chr6:117645578 | *CD74*(6)-*ROS1*(34) | 28678 | IHC |
| 7 | chr2:42552694-chr2:29446394 | *EML4*(20)-*ALK*(20) | 4353 | IHC |
| 8 | chr10:32311068-chr2:29446394 | *KIF5B*(17)-*ALK*(20) | 9641 | IHC |
| 9 | chr5:149784243-chr6:117645578 | *CD74*(6)-*ROS1*(34) | 4347 | IHC |
| 10 | chr2:42522656-chr2:29446394 | *EML4*(13)-*ALK*(20) | 5821 | IHC |
| 11 | chr2:42522656-chr2:29446394 | *EML4*(13)-*ALK*(20) | 2279 | IHC |
| 12 | chr2:42522656-chr2:29446394 | *EML4*(13)-*ALK*(20) | 1659 | IHC |
| 13 | chr2:42492091-chr2:29446394 | *EML4*(6)-*ALK*(20) | 349 | IHC |
| 14 | chr2:42491871- | *EML4*(6)- | 4401 | IHC |

| | | | | |
|---|---|---|---|---|
| | chr2:29446394 | *ALK*(20) | | |
| 15 | chr2:42543190-chr2:29446463 | *EML4*(18)-*ALK*(20) | 460 | IHC |
| 16 | chr2:42552694-chr2:29446394 | *EML4*(20)-*ALK*(20) | 12782 | IHC |
| 17 | chr2:42522656 - chr2:29446394 | *EML4*(13) - *ALK*(20) | 4145 | NGS |
| 18 | - | Not detected | - | FISH |
| 19 | unknown | *ALK* (unknown partner) | - | FISH |
| 20 | chr2:42492091 - chr2:29446394 | *EML4*(6) - *ALK*(20) | 16459 | FISH plus IHC |
| 21 | chr2:42491871 - chr2:29446394 | *EML4*(6) - *ALK*(20) | 6005 | FISH plus IHC |
| 22 | chr2:42522656 - chr2:29446394 | *EML4*(13) - *ALK*(20) | 6079 | FISH |
| 23 | chr10:61665880 - chr10:43612032 | *CCDC6*(1) - *RET*(12) | 27834 | NGS |
| 24 | - | Not detected | - | FISH |
| 25 | unknown | *ALK* (unknown partner) | - | FISH |
| 26 | chr2:42491871 - chr2:29446394 | *EML4*(6) - *ALK*(20) | 317 | FISH plus IHC |
| 27 | chr2:42522656 - chr2:29446394 | *EML4*(13) - *ALK*(20) | 9513 | NGS |
| 28 | chr4:25678324 - chr6:117650609 | *SLC34A2*(13) - *ROS1*(32) | 194178 | NGS plus FISH plus IHC |
| 29 | chr1:154142878 - chr6:117642559 | *TPM3*(8) - *ROS1*(35) | 1211 | FISH |
| 30 | chr7:75172170 - chr2:29446394 | *HIP1*(28) - *ALK*(20) | 24768 | FISH plus IHC |
| 31 | chr2:42543190 - chr2:29446394 | *EML4*(18) - *ALK*(20) | 19127 | FISH plus IHC |

| | | | | |
|---|---|---|---|---|
| 32 | - | Not detected | - | NGS |
| 33 | chr2:42491871 - chr2:29446394 | *EML4*(6) - *ALK*(20) | 1587 | IHC |
| 34 | chr5:149784243 - chr6:117645578 | *CD74*(6) - *ROS1*(34) | 5340 | FISH plus IHC |
| 35 | chr10:32317356 - chr10:43612032 | *KIF5B*(15) - *RET*(12) | 29390 | NGS |
| 36 | unknown | *ALK* (unknown partner) | - | FISH plus IHC |
| 37 | - | Not detected | - | NGS |
| 38 | - | *MET* exon 14 skipping | - | NGS |
| 39 | chr2:42522656 - chr2:29446394 | *EML4*(13) - *ALK*(20) | 50661 | NGS |
| 40 | chr2:42522656 - chr2:29446394 | *EML4*(13) - *ALK*(20) | 2385 | NGS |
| 41 | - | *MET* exon 14 skipping | - | NGS |
| 42 | chr2:42522656 - chr2:29446394 | *EML4*(13) - *ALK*(20) | 10836 | NGS |
| 43 | chr10:61665880 - chr10:43612032 | *CCDC6*(1) - *RET*(12) | 15342 | NGS |
| 44 | chr10:61665880 - chr10:43612032 | *CCDC6*(1) - *RET*(12) | 24018 | NGS |
| 45 | chr2:42552694 - chr2:29446394 | *EML4*(20) - *ALK*(20) | 11823 | FISH plus IHC |
| 46 | chr2:42472827 - chr2:29446394 | *EML4*(2) - *ALK*(20) | 53460 | NGS plus IHC |
| 47 | - | *MET* exon 14 skipping | - | NGS |
| 48 | - | Not detected | - | FISH plus IHC |

Abbreviations: *ALK*: Anaplastic Lymphoma Kinase; *CCDC6*: Coiled-Coil Domain Containing 6; *CD74*: CD74 Molecule, Major Histocompatibility Complex, Class II Invariant Chain; chr: chromosome; *EML4*: Echinoderm Microtubule Associated Protein Like 4; F: female; FISH: fluorescent in situ hybridization; *HIP1*: Huntingtin Interacting Protein 1; IHC: immunohistochemistry; *KIF5B*: Kinesin Family Member 5B; M: male; *MET*: MET Proto-Oncogene, Receptor Tyrosine Kinase; NGS: next generation sequencing; *RET*: Rearranged During Transfection; *ROS1*: ROS Proto-Oncogene 1, Receptor Tyrosine Kinase; *SLC34A2*: Solute Carrier Family 34 Member 2; *SDC4*: Syndecan 4; *TPM3*: Tropomyosin 3.

## 5 - Discussion

In the era of personalized medicine is mandatory to test a crescent number of approved biomarkers to ensure the best therapeutic choice for NSCLC patients. In this scenario, the emerging role that several fusion events have rapidly acquired in the management of NSCLC patients lead to the identification of major criticisms related to the implementation of molecular tests in diagnostic routine. First, scant starting material, generally represented by an unique diagnostic slide or a small biopsy, is inadequate to satisfy all the molecular requirements for the clinical management of lung cancer patients.[32] Second, single test methodologies are associated with a higher risk to consume all the available diagnostic tissue material if an extensive molecular analysis is carried out. To date, IHC and FISH represent the gold standard technical approaches for the identification of gene fusions (*ALK*, *ROS1*, *RET* and *NTRK*) in the diagnostic routine setting but several limitations affect their employment in the field of personalized medicine, in particular a major issue is represented by a not negligible percentage of discordant cases between the two methodologies.[38] Another unsolved question regards the misinterpretation of a positive results if breakpoint falls in a genomic region not adequately covered by a specific probe, in addition designed probes are generally not able to identify less common fusion events that could predict a positive response to TKIs for NSCLC patient.[39] At the light of these elements, multi test technology are rapidly changing the technical management of molecular testing. Currently, NGS is the most attractive and fascinating technology that may be approached to resolve these technical issues.[40] Several NGS panels are commercially available to identify a wide range of molecular alteration in solid tumors, including fusion events but technical performance is strictly dependent from the quality and quantity of starting material. On the basis of this evidence, we have introduced narrow NGS panel that allows to analyse all the clinically relevant mutations in scant diagnostic samples showing how a tailored panel for lung cancer alterations may improve analytical performance in clinical practice. On the basis of this experience, we have developed a custom NGS panel for RNA fusion analysis that may integrate DNA analysis of diagnostic routine samples. In our experience, we demonstrated that an

ultra-deep sequencing approach by using our custom narrow SiRe NGS fusion panel for the detection of clinically relevant gene fusions and plus *MET* exon 14 skipping alteration was useful on lung cancer routine samples. In addition, our approach was able to identify all gene fusions and the splicing event harbored by RNA pool extracted from cell lines even when the RNA concentration was of 2 ng/µl, that is very closed to the quantity of RNA obtained from cytological samples and small histological biopsy, that represent the 80% of NSCLC sample types in the advanced stage setting. Finally, we can confirm that the SiRe gene fusion panel has a high analytical and clinical performance to detect the gene fusion and *MET* exon 14 skipping alteration to select NSCLC patients to TKI's therapy.

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