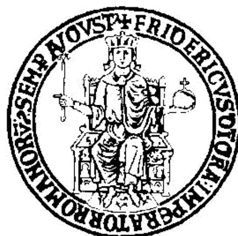
UNIVERSITA' DEGLI STUDI DI NAPOLI FEDERICO II



DIPARTIMENTO DI SANITA' PUBBLICA

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

"Application of Next Generation Technologies in liquid biopsy: focus on Non Small Cell Lung Cancer and Metastatic Colorectal Cancer"

Tutor:

Ch.mo Prof. Giancarlo Troncone

Candidato:

Dott. Francesco Pepe

Coordinatore:

Ch.mo Prof. Stefania Montagnani

ANNO ACCADEMICO 2017- 2018

Abstract……………………………………………………………………4

Introduction 6

Chapter 1

1.1 : Liquid biopsy based Coloscape™ assay evaluation in triage of fit+ patients …………………………………………………………..9

1.1.1 : Material and Methods……………………………………………10

1.1.2 : Results 13

1.1.3 :Discussion.. 15

Chapter 2

2.1 : Development of a gene panel for next generation sequencing of clinically relevant mutations in cell-free DNA from cancer patients...................17

2.1.1 : Material and Methods 18

2.1.2 : Results 25

2.1.3 :Discussion.. 31

Chapter 3

3.1 : Cell free DNA analysis by SiRe® next generation sequencing panel in non small cell lung cancer patients: focus on basal setting 35

3.1.1: Material and Methods...36

3.1.2: Results 38

3.1.3:Discussion.. 43

References 45

Background: In the precision medicine era, the increasing request of clinical relevant biomarkers to improve the patients management lead to the need of most biological source. To address this issue, also if tissue represents the gold standard for the assessment of clinical relevant biomarkers mutational status, some alternative approaches, based on the analysis of circulating free DNA (cfDNA) extracted from “liquid biopsies”, are under evaluation. The aims of this thesis were to investigate the role of liquid biopsy in two specific settings: analysis of EGFR mutational status in Non Small Cell Lung Cancer (NSCLC) patients treated with EGFR Tyrosine Kinase Inhibitors (TKIs) and as a screening tool for Colorectal Cancer (CRC) in comparison of Fetal Immunochemical Test (FIT).

Methods: Regarding EGFR mutational status assessment in NSCLC patients, the analytical sensitivity of SiRe panel, which covers 568 mutations in six genes (EGFR, KRAS, NRAS, BRAF, cKIT and PDGFRα) was validated on cell line DNA and cfDNA derived from cancer patients at presentation (n=42), treatment response (n=12) and tumor progression (n=11) were analyzed; all patients had paired tumor tissue and cfDNA previously genotyped with a Taqman-derived assay (TDA). In addition, we tested blood samples prospectively collected from NSCLC patients (n=79) to assess the performance of SiRe in clinical practice.

In relation to CRC patients, employing the analytical validated Real Time PCR-based ColoScape assay kit, mutations in the APC, KRAS, BRAF and CTNNB1 genes were assessed on 52 prospectively collected whole-blood samples obtained from FIT+ patients enrolled in the CRC screening program of ASL NAPOLI 3 SUD, using colonoscopy as confirmation.

Results: In relation to the analysis of EGFR mutational status in NSCLC patients treated with EGFR TKIs, SiRe showed high analytical performance and a 0.01% lower limit of detection. Regarding the results obtained in the retrospective series, SiRe was able to detect 40 EGFR, 11 KRAS, 1 NRAS and 5 BRAF mutations (96.8% concordance with TDA). In the baseline sample set, SiRe had 100% specificity and 79% sensitivity relative to results obtained on paired tumor

tissue. In the prospective series, SiRe detected 8.7% (4/46) of EGFR mutations at baseline and 42.9% (9/21) of EGFR p.T790M in patients at tumor progression.

Regarding the application of Real Time PCR based ColoScape assay kit as a screening tool for CRC patients, the assay's sensitivity for advanced adenomas was 53.8% and the specificity was 92.3%. The Positive Predictive Value was 70.0% and negative predictive value was 85.7%. Of note, four of the six positive cases missed by ColoScape had a less than suboptimal DNA input. Had they been ruled out as inadequate, sensitivity would have increased from 53.8% to 69%.

Conclusions: In the landscape of EGFR mutated NSCLC patients treated with TKIs, SiRe represents a feasible NGS panel for cfDNA analysis in clinical practice, while in CRC patients setting, ColoScape is a promising tool for screening program aims to evaluate the triage of FIT+ patients.

Introduction

In the precision medicine era, the increasing request of clinical relevant biomarkers improves the importance of patients management giving the opportunity to realize a “tailed therapy” based on molecular features of neoplastic disease for each tumor patients.(1) Several type of specimens are adopted to provide mutational assessment of clinical relevant biomarkers for each patient but independently from sample type (cytological, histological) and sample preparation (FNA, liquid based cytology, cell block, FFPE) increasing number of clinical biomarkers revealed the need of a biological source characterized by high quality and quantity to perform molecular tests.(2) Several limitations affect the use of tissue specimen in clinical setting: the discomfort suffered by the patient, clinical risks, tumor heterogeneity, potential surgical complications and economic considerations meaning that multiple or serial biopsies are often impractical.(3) To address this issue, also if tissue represents the gold standard for the assessment of clinical relevant biomarkers mutational status, some alternative approaches, based on the analysis of circulating free DNA (cfDNA) extracted from “liquid biopsies”, are under evaluation. Indeed, the specific detection of tumor-derived cfDNA has been shown to correlate with tumor burden, to a change in response to treatment or surgery, to indicate that subpopulations of tumor cells acquired resistance to a specific treatment and to represent a prognostic tool in relation to selected molecular features.(4)

The aims of this thesis were to investigate the role of liquid biopsy in two specific settings: analysis of EGFR mutational status in Non Small Cell Lung Cancer (NSCLC) patients treated with EGFR Tyrosine Kinase Inhibitors (TKIs) and as a screening tool for Colorectal Cancer patients (CRC) in comparison of Fetal Immunochemical Test (FIT). In the first chapter of this thesis the prognostic role of liquid biopsy in CRC patients was evaluated. Today Screening programs for colorectal cancer in Europe are based on FIT test as a primary screener. FIT+ patients are referred to immediate colonoscopy and the PPV is usually 25%. Liquid biopsy may be introduced in this screening setting by replacing Fit test as a primary screening tool in attemption

to reduce patients with adenoma who referred to colonoscopy. (5-6) In 2017, DiaCarta Inc., a company based in Richmond (California), developed ColoScape^{TM}, a RT-qPCR based assay that exploits wild-type clamping probe technology to amplify selectively mutated DNA, was investigated in order to evaluate technical performance in a retrospective sample setting of FIT+ patients.

The second chapter focused on predictive role of liquid biopsy about clinical relevant mutation detection in lung cancer patients. In addition to EGFR, EMEA and AIFA approved analysis of other relevant biomarkers (ALK, ROS-1, RET, and PDL-1), in clinical practice for lung cancer patients to offer a complete molecular profile of genetic alterations sensitizing to small molecules thyrosine kinase inhibitors (TKIs) Gefitinib (Iressa®, AstraZeneca, London, UK) and Erlotinib (Tarceva®, F. Hoffmann-La Roche, Basel, Switzerland), or the second-generation TKI Afatinib (Giotrif®, Boehringer Ingelheim, Ingelheim, Germany).(7) In a wide range of lung cancer patients the biological source is represented by a unique cytological slide on which morphological diagnosis and molecular tests could be performed. In 16% of cytological specimens nucleic acids quantity extracted can not reached DNA input required to perform molecular tests; in this case liquid biopsy may be applied to detect sensitizing mutation in EGFR and predict clinical response to TKIs in NSLC patients according to histological type (adenocarcinoma), sex, smoking history, histological grade or other clinical risk factors. (8) To evaluate circulating free DNA (cfDNA) is necessary the implementation in clinical setting of next generation technologies characterized by high sensitivity and specificity in order to detect also <1% clinical relevant mutations.(9) As it is detailed in chapter 2, this thesis describes the implementation of a custom NGS panel able to detect 568 clinical relevant mutations in six genes which play a predictive role in four solid tumors and analyzes the performance of this panel in a prospective clinical group following clinical parameters, progression free survival (PFS) and overall survive (OS), in a specific subgroup.

The third chapter addressed the implementation of liquid biopsy in clinical practice of Predictive Molecular Lab of University Federico II to evaluate clinical relevant mutations in predictive biomarkers of NSCLC patients by NGS platform. Cell-free DNA (cfDNA) can be used as a surrogate for *EGFR* mutational testing, whenever tissue is unavailable. However, the detection of gene mutations on cfDNA is challenging; in fact, the extremely low concentration of circulating tumor DNA requires the implementation of highly sensitive and validated next generation techniques. (10)

Chapter 1

1.1 Liquid biopsy based Coloscape^{TM}assay evaluation in triage of fit+ patients.

Colorectal cancer is the third most frequent cancer in the world. Approximately 1.7 million new cases were diagnosed in 2015, with about 832,000 deaths. The progression from pre-cancer to cancer and metastasis is relatively slow, averaging 15 years. This creates an opportunity for early detection and successful treatment. In Europe, the test of choice in most screening programs is the fecal immunochemical test for the detection of blood in the stool (FIT).(5) Patients who test positive at FIT are referred to colonoscopy, where, however, about 75% of them turn out to be negative.(6) An intermediate test with good sensitivity and specificity could help select FIT+ patients at greater risk to be positive at colonoscopy. Researchers all over the world have focused their attention on mutational analysis with a view to identifying biomarkers that could aid in the early detection of CRC and/or its recurrences. Some important results have been obtained in late stage and metastatic cancer, where mutational analysis is now routinely used prior to prescribing some novel biological therapies.(11) The assessment of wild-type status in the RAS gene is a prerequisite to the use of cetuximab and panitumumab, to give an example.(12) On the other hand, not much experience and literature exist on molecular analysis in early detection of CRC. An article published in the NEJM in 2014 (13) described an FDA-approved stool-DNA test (Cologuard, Exact Sciences, Madison, WI) and reported sensitivity of 42% for advanced adenomas and 92% for cancer, with a specificity of 87%. Other work has been done employing Septin 9 (Epigenomics), another FDA-approved test based on detection of methylation markers in

blood samples.(14) In 2017, DiaCarta Inc., a company based in Richmond (California), developed ColoScape^{TM}, an assay that combines a multiplex gene biomarker panel developed by Dr Bettina Scholka at the University of Postdam (Germany)(15-16) with proprietary xenonucleic acid (XNA) wild-type clamping probe technology. XNA allows the selective DNA polymerase amplification of only target nucleic acid templates that contain mutations, while blocking wild-type templates, thus maximizing analytical sensitivity. In this preliminary pilot study the sensitivity and specificity of the ColoScape^{TM} assay were investigated in order to collect some initial performance parameters as a basis to design a follow-on study of adequate power and sample size that will provide information for the assay's potential use in the triage of FIT+ patients.

1.1.2 Material and Methods

Patient and sample collection

Sixty patients referred to colonoscopy for a FIT+ test were enrolled by the Gastroenterology Department of ASL Napoli 3 Sud – Hospital S. Maresca of Torre del Greco. Informed consents were obtained and 20 ml of blood were drawn from each patient and stored in Cell-Free DNA BCT® Streck tubes.

Plasma separation and DNA extraction

Whole-blood samples were transferred to the processing laboratory (Predictive Molecular Pathology Laboratory, Department of Public Health, University Federico II of Naples), where the plasma was separated using the previously described double-spin.(17) Approximately 10 ml of plasma were obtained from each sample and frozen for later use. Cell-free DNA (cfDNA) was extracted using QIAamp Mini Elutec cfDNA Kit (QIAGEN, Hilden, Germany) according to the

manufacturer's instructions. Evaluation of DNA quality and quantity was performed on TapeStation 4200 (Agilent, Santa Clara, California, USA).

Coloscape^{TM}assay test

The ColoScape^{TM} kit (DiaCarta Inc., Richmond, CA), is a real-time PCR based in vitro diagnostic assay for the detection of colorectal cancer associated mutations in genes including APC (codons 1309,1367,1450,) KRAS (codons 12 and 13), BRAF (codon 600) and CTNNB1 (codons 41 and 45).(15) The assay can be performed on DNA extracted from either formalin-fixed paraffin-embedded (FFPE) or plasma samples to identify the presence or absence of mutations in the targeted regions but does not specify the exact nature of the mutation. The QClamp technology used by the ColoScape^{TM} assay is based on XNA mediated PCR clamping technology. XNA is a synthetic DNA analog in which the phosphodiester backbone has been replaced by a novel synthetic backbone chemistry. XNAs hybridize tightly to complementary DNA target sequences only if the sequence is a complete match. Binding of XNA to its target sequence blocks strand elongation by the DNA polymerase. When there is a mutation in the target site, and therefore a mismatch, the XNA-DNA duplex is unstable, allowing strand elongation by the DNA-polymerase. Addition of an XNA, whose sequence is a complete match to the wild-type DNA, to a PCR reaction, blocks amplification of wild-type DNA allowing selective amplification of mutant DNA(18). XNA oligomers are not recognized by DNA-polymerases and cannot be utilized as primers in subsequent real-time PCR reactions. (Figure 1)

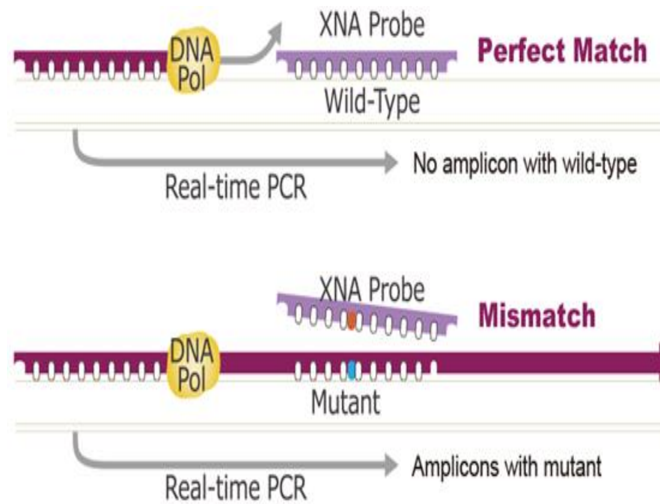


Figure 1. The QClamp technology used by the ColoScape™ assay. XNA is a synthetic DNA analog that hybridizes tightly to complementary DNA target sequences only if the sequence is a complete match. When there is a mutation in the target site, and therefore a mismatch, the XNA-DNA duplexis unstable, allowing strand elongation by the DNA-polymerase.

The test was performed on ABI QuantStudio 5 instrument according to DiaCarta's instructions and the cycling parameters were presented in Table 1.

Step	Temperature (°C)	Time (Seconds)	Ramp Rate (°C/s)	Cycle	Data Collection
Pre incubation	95	300	1.6	1	OFF
Denaturation	95	20	1.6	X50	OFF
XNA Annealing	70	40	1.6		OFF
PrimerAnnealing	66	30	1		OFF
Extension	72	30	1		FAM and VIC

Table 1. ColoScape™ cycling parameters on ABI QuantStudio 5.

1.1.3 Results

cfDNA was successfully extracted from all the samples and no genomic DNA contamination was observed based on TapeStation analysis (data not shown). The estimated cfDNA concentrations varied largely ranging from 0.4 to 9.0 ng/µL and, as expected, the extracted cfDNA concentrations from 10 mL plasma were much higher than those from 5 mL plasma (median 2.9 vs 1.6 ng/µL). There were 52 valid samples and 8 samples were excluded from analysis due to either a missing colonoscopy report or technical reasons. Advanced precancerous lesions (AA) include all advanced adenomas and sessile serrated polyps measuring 1 cm or more in size. No cancers were found in this sample set. Colonoscopy was used as the truth throughout to calculate performance indicators. Out of 52 valid samples, 13 showed positive colonoscopy results among which 7 were tested positive by ColoScape™ assay with a sensitivity being 53.8%. Among 39 samples with negative colonoscopy results, 36 samples were tested as negative by ColoScape™ assay with a specificity being 92.3% (Tables 2).

	Colonoscopy positive	**Colonoscopy negative**	**Total**
ColoScape™ positive	7	3	10
ColoScape™ negative	6	36	42
Total	13	39	52

Table 2. Summary of Colonoscopy and ColoScape™ results

The results of 10 samples tested positive by ColoScape™ assay were presented in Table 3.

Sample ID	Colonoscopy results	ColoScape^{TM} Results
18	positive	KRAS 12 positive
19	positive	APC 1450 positive
24	positive	KRAS 12 positive
28	negative (1 polyp of 4 mm not meeting positivity criteria)	KRAS 12 strong positive (KRAS c.35G>A; p.G12D confirmed by Sanger sequencing)
35	negative	APC 1450 positive
40	negative	KRAS 12 positive
45	positive	KRAS 12 & BRAF 600 positive
50	positive	CTNNB1 45 positive
54	positive	APC 1450 positive
55	positive	KRAS 12 positive

Table 3. Results of 10 samples tested positive by ColoScape^{TM} assay

1.1.4 Discussion

Liquid-biopsy is a challenging type of sample for mutational analysis. We look for cfDNA and ctDNA. Estimates for ctDNA range from 1 to 10% of cfDNA. On top of this, mutations can occur at different allelic frequencies, which may be in some cases as low as 0.1%.(19) XNA aims to maximize analytical sensitivity due to its ability to selectively amplify only, or predominantly, mutant forms and block wild-types. The manufacturer recommends a minimum of 5 ng of DNA per reaction, although there are evidences that it could work with a 2.5 ng DNA input as well. In this pilot study, it was aimed to assess the limits of the assay considerably, and determined to accept even samples with a sub-optimal DNA input, for the goal was to establish the best work-flow for advanced adenomas. Of note, 4 of the 6 positive cases missed by ColoScape™ had a less than suboptimal DNA input (data not shown). Had they been ruled out as inadequate, sensitivity would have increased from 53.8 to 69%. However, as stated previously , this is not a clinical trial, but rather an initial, preliminary technical evaluation. The most prevalent mutation was found in the KRAS gene (4 cases). Other mutations were APC (2 cases) and CTNNB1 (1 case), and BRAF in one case of dual positivity with KRAS. Interestingly, a case (#28) of a polyp with size of 4 mm, which did not meet the positivity criteria, showed a KRAS positivity and Sanger sequencing confirmed the mutation being KRAS c.35G>A; p.G12D. One case that was excluded due to inadequate bowel preparation, was negative and showed no relevant genetic variations.

Given the small sample size, sensitivity, specificity and resulting predictive values, must be considered only estimates that will help design and power a future clinical trial. However, it is of considerable interest to consider that detection of advanced adenomas is a real challenge for screening programs that are based on the FIT test, and for the other clinically approved molecular tests, such as Cologuard and Septin 9. One has to also consider specificity that should ideally exceed 90% in order to rule out a significant number of FIT+ patients that now turn out negative on colonoscopy. This pilot study justifies further investigation of the ColoScape™ assay. The

most important result obtained from this study was the identification of a clinically relevant work-flow that can optimize performance and allows an estimation of the test sensitivity and specificity that will be a crucial focus of the future trial. Other interesting aspects to be investigated will be: management of FIT+, triage – patients, management of FIT+, triage + and colonoscopy – patients, management of patients with inadequate bowel preparation. Based on the results from this study, it

further studies are warranted in order to validate the use of liquid biopsy – based ColoScape^{TM} assay for the triage of FIT+ patients.

Chapter 2

2.1. Development of a gene panel for next generation sequencing of clinically relevant mutations in cell-free DNA from cancer patients.

Precision medicine, coupled with the tissue-based assessment of biomarkers predictive of treatment outcome, has transformed pathology practice. (20) RAS and BRAF mutation testing in colorectal cancer (21,22), EGFR in non-small-cell lung cancer (NSCLC, 23) BRAF in melanoma (24) and cKIT and PDGFRa in gastrointestinal stromal tumours (25) has added a genotypic element to the phenotypic diagnostics of solid tumours. However, tumour tissue is not always available or may be insufficient for molecular testing. In this setting serum- and plasma-derived cfDNA represents a complementary biological source to evaluate molecular assessment of clinical relevant biomarkers by adopting next generation sequencing platforms (NGS), which can be multiplexed across several genes to cover less common and even novel variants (26, 27). Large gene panels or whole-exome approaches to screen for a large number of genomic regions may not be easily implemented in cfDNA analysis , in fact circulating tumour DNA represents only a small fraction (<0.5%) of the total cfDNA and a 'ultra-deep sequencing' strategy, based on implementation of small NGS panels that tailored to target a limited number of actionable genes, can significantly increase analytic sensitivity reducing the number of samples classified as "inadequate". Following this concept Molecular pathology lab of University Federico II designed and developed a narrow gene panel that targets 568 clinically relevant mutations in six genes (EGFR, KRAS, NRAS, BRAF, cKIT and PDGFRa) involved in non Small cell lung cancer, gastroIntestinal stromal tumour, metastatic coloRectal carcinoma and mElanoma (whose acronym is SiRe) starting from mucleic acids derived from

tissue sample or liquid biopsy with high sensitivity and specificity. Moreover, its clinical performance in a clinical setting was evaluated.

2.1.1 Materials and Methods

Design of the SiRe panel.

The Ion AmpliSeq Designer suite v5.3.1 with hg19 was used as reference genome to develop a customised panel targeting six genes (EGFR, KRAS, NRAS, BRAF, cKIT and PDGFRa) that are associated with treatment outcome in NSCLC, GIST, CRC and metastatic melanoma (21-24). A single primer pool leading to the selection of 42 amplicons (ranging from 125 to 175bp) enabled us to cover all COSMIC annotated mutations (n=568) in the selected exons of the target genes. The amplicon design covering 5.2kb of genomic DNA was optimized for the simultaneous analysis of 16 samples with the 316v2 chip (Thermofisher, Foster City, CA, USA) on a Personal Genome Machine Torrent (Thermofisher).

Study design, patients and samples.

The panel performance was evaluated in three steps (Figure 2). First, the analytical sensitivity of the assay was assessed on DNA from two cell lines and by using an artificial reference standard with multiple mutations in different genes. Second, clinical sensitivity and specificity was determined using archival cfDNA from 63 cancer patients with paired tumour tissue, previously genotyped with a Taqman derived assay(TDA). As exploratory analysis, to confirm that our NGS approach cover the mutations in cKit and PDGFRa genes, two GIST samples (bloods and tissues) were tested with SiRe and the relative data are not reported only in this thesis. Third, the performance of the panel in daily clinical practice was assessed using blood samples prospectively collected from patients with advanced NSCLC. Written informed

consent was obtained from all patients and documented 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 Helsinki Declaration (http://www.wma.net/ en/30publications/10policies/b3/). DNA purification. DNA from the two cell lines was isolated using the QIAamp Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Circulating-free DNA was purified as follows: 15ml blood was withdrawn from patients and collected in Vacutainer tubes (BD, Plymouth, UK). Plasma and serum were isolated by centrifugation twice at 2300 r.p.m. for 10min. The supernatant (serum or plasma) was aliquoted and used immediately for cfDNA isolation or stored at - 80ºC. Cell-free DNA was purified from serum and plasma for each patient (1.2ml). In the rare instances that the volume of the serum and plasma sample obtained from a patient was between 1 and 1.2ml, PBS up to 1.2ml was added to the samples, which were then purified using the QIAsymphony robot (Qiagen) and the QIAsymphonyDSPVirus/ Pathogen Midi Kit, according to the manufacturer's instructions, and cfDNA was eluted in a final volume of 30ml. Since correct pre-analytical handling of blood specimens is crucial to maintain the sample informative, the process was standardised (in terms of blood collection, sample centrifugation and cfDNA extraction) in the Department of Public Health of the University of Naples Federico II, and all procedures were performed in-house by a nurse belonging to the laboratory staff.

Sample sequencing.

We analysed the serum and plasma cfDNAs of each patient enrolled in the study. Libraries were constructed and purified on the Ion Chef (Thermofisher), and eight samples (corresponding to 4 patients) were added per run. Library generation was as follows: 15μ of cfDNA were dispensed on Ion Code plates and amplified using Ion AmpliSeq DL8 (Thermofisher). We used 22 cycles for cfDNA amplification and 6 cycles for library reamplification after barcoding, under the thermal conditions defined by the manufacturer. Purified libraries derived from eight cfDNA samples were diluted to 60pM and combined with eight additional cfDNA-derived libraries to obtain a 16 Ion Code pooled library. The two-pooled libraries were re-loaded into the Ion Chef instrument, and templates were prepared using the Ion PGM Hi-Q IC Kit (Thermofisher). Finally, templates were loaded into the 316v2 chip and sequenced on PGM. Data analysis. Signal processing and base calling were carried out using the default base-caller parameters on Torrent Suite [v.5.0.2] and coverage analysis was performed using SiRe designed bed files with coverage plug-in (v.5.0.2.0). BAM files were visually inspected with the Golden Helix Genome Browser v.2.0.7 (Bozeman,MT, USA). Variants were automatically annotated using variant caller plug-in (v.5.0.2.1) at specific optimised parameters of the SiRe panel. In particular, only variants with ≥5X allele coverage and a quality score ≥20, within an amplicon that covered at least 1000X alleles, were called, and the frequency of each mutant allele was recorded.

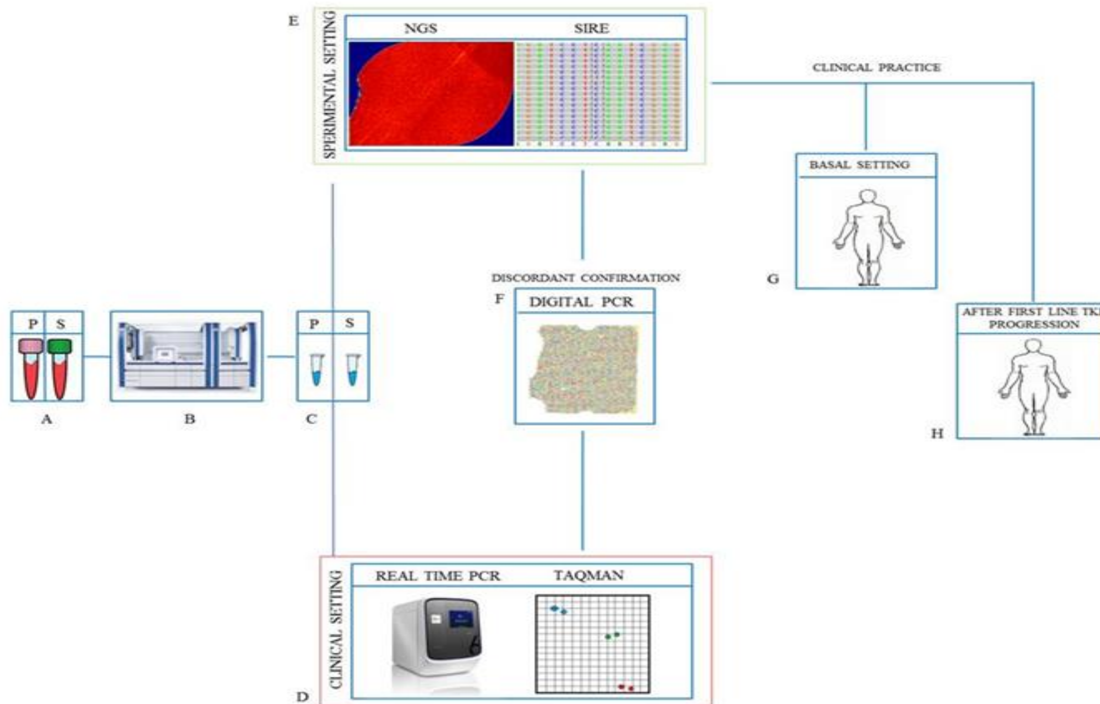


Figure 2. **Study design .**cfDNAs (A) extracted with the QIAsymphony virus/pathogen kit (B) from paired (P) plasma and (S) serum (C) samples were analyzed by quantitative 50-nuclease TaqMan PCR (D) and by the NGS SiRe panel (E). Any discordance between the two techniques was evaluated by dPCR (F). After preclinical validation, the SiRe panel was applied in clinical practice in cases in which tissues were not available to select patients for TKI treatment, at baseline (G), and to evaluate the selection of resistant clones after disease progression (H). (ref. 17)

Preclinical assessment.

Genomic DNA from the HCC827 (EGFR p.E746-A750del; KRAS wt) and A549 (EGFR wt; KRAS p.G12S) cell lines was used to assess analytical performance. Both cell lines were obtained from the National Research Council/Institute of Experimental Endocrinology and Oncology on courtesy of Dr Pierlorenzo Pallante (Naples, Italy). The analytical sensitivity of the assay for point mutation and indel detection was determined by diluting DNA from the appropriate mutated cell line (A549 for point mutations and HCC827 for indels) into increasing concentrations of DNA from the appropriate wt cell line (HCC827 for point mutations and

A549 for indels). DNA dilutions ranged between 1:10 and 1:10000, which correspond to allelic fractions from 1:20 to 1:20000 of the mutated allele (both cell lines are heterozygous). Each dilution was analyzed in duplicate to estimate inter-run assay reproducibility, and the library obtained from each dilution was sequenced twice to evaluate intra-run assay reproducibility. In addition, customized Horizon Diagnostics Multiplex gDNA reference standard, with mutation in EGFR (p.E746_A750del and p.G719S), KRAS (p.G12D), NRAS (p.Q61L) and BRAF (p.V600E), each of them at three different dilution points (1, 0.5 and 0.1%), were assessed to provide stronger evidence on SiRe analytical performance.

Clinical validation.

We determined the specificity and sensitivity of our assay by analyzing archival serum and plasma cfDNA from 40 cancer patients at presentation attending the QuironDexeus University Hospital (33 NSCLC, 2 CRC and 5 metastatic melanoma) with paired tumour tissue. In addition, we tested archival serum and plasma cfDNAs from 12 responder patients and 11 patients at the time of tumour progression after treatment (18 NSCLC, 2 CRC and 3 metastatic melanoma; Table 4). All of the 63 cfDNA samples and tumour tissues had previously been genotyped for EGFR, KRAS, NRAS and BRAF mutations using a TDA (28, 29). In the case of tumour tissues, genotyping had been confirmed by standard PCR followed by Sanger sequencing. Cases showing discordance between the NGS SiRe panel and the TDA were further investigated by digital PCR (dPCR) on a QuantStudio 3D Digital PCR System platform (Thermofisher) as previously described. (30)

Performance of the SiRe panel in prospective clinical samples.

To evaluate the performance of the SiRe panel in the clinical setting, we prospectively

genotyped 79 advanced NSCLC patients (37 men and 42 women; mean age: 65 years) using blood samples collected at the Department of Public Health of the University of Naples Federico II (Table 4). According to the European Medicines Agency guidelines, mutations related to EGFR disease were tested in patients when tissue was not available at presentation (n=46), or at tumour progression (n=33) in patients previously treated with erlotinib (n=14), gefitinib (n=14) or afatinib (n=5) in the attempt to detect the emergence of resistance secondary mutations. In 21 of the 33 cases with tumour progression, first-line TKI administration had been based on the demonstration of an EGFR mutation in tissue, whereas in the remaining 12/33 cases, TKI treatment had been administrated in second line without evidence of EGFR mutations.

	Clinical characteristics	**Retrospective validation (*N*=63)**	**Prospective validation (*N*=79)**
Age			
	≥29–60	22 (34.92%)	22 (27.85%)
	<61–80	25 (39.68%)	
	Unknown	16 (25.40%)	57 (72.15%)
Sex			
	Male	24 (38.10%)	37 (46.84%)
	Female	24 (38.10%)	42 (53.16%)
	Unknown	15 (23.80%)	
Smoking status			
	Never smokers	11 (17.46%)	38 (48.10%)
	Ex-smokers	9 (14.30%)	29 (36.70%)
	Smokers	5 (7.93%)	6 (7.60%)
	Unknown	38 (60.31%)	6 (7.60%)

Type of tumour			
	Lung Colorectal carcinoma Metastatic melanoma	51 (80.95%) 4 (6.35%) 8 (12.70%)	79 (100%)
Stage			
	IIIB–IV Unknown	48 (76.20%) 15 (23.80%)	79 (100%)
Histology			
	Adenocarcinoma Large cellcarcinoma Undifferentiated carcinoma Metastatic melanoma Unknown	35 (55.55%) 1 (1.60%) 4 (6.35%) 8 (12.70%) 15 (23.80%)	79 (100%)
Somaticalterations			
	EGFR mutations KRAS mutations BRAF mutations NRAS mutations No mutations	32 (50.79%) 15 (23.80%) 7 (11.11%) 1 (1.60%) 8 (12.70%)	25 (31.65%)
Type of sample			
	Pretreatment Response evaluation TKIs Chemotherapy Progressive disease TKIs Chemotherapy	40 (63.50%) 12 (19.04%) 8 (66.70%) 4 (33.30%) 11 (17.46%) 9 (81.81%) 2 (18.19%)	46 (58.23%) 33(41.77%) 33 (41.77%)

Table 4. Characteristics of the patients included in the retrospective (left) and prospective (right) clinical validation of the SiRe panel

2.1.2 Results

Panel design and preclinical performance evaluation.

The SiRe panel was designed to cover 568 clinically relevant mutations in six genes (EGFR, KRAS, NRAS, BRAF, cKIT and PDGFRa) involved in NSCLC, GIST, CRC and metastatic. In Preclinical evaluation, in On cell line derived DNA, the SiRe panel detected the EGFR deletion p.E746_A750del and the KRAS point mutation p.G12S at a level as low as one copy of the mutated allele in a background of 20000 copies of wild-type alleles (0.005% mutated allele fraction), with 100% of intra- and inter-run reproducibility. In addition, regarding the results obtained on multiplex gDNA reference standard (Horizon Diagnostics), p.E746_A750del and p.G719S point mutation in EGFR, p.G12D mutation in KRAS exon 2, p.Q61L mutation in NRAS exon 3 and p.V600E mutation in BRAF exon 15 were correctly identified for each different dilution point. This high analytical performance was achieved thanks to the use of optimised parameters set in variant caller plug-in (v.5.0.2.1) which detected low abundant mutated alleles with a specificity of 100%.

Clinical sensitivity and specificity of the SiRe panel in cfDNA samples.

The retrospective series of cfDNAs was constituted by 126 paired serum and plasma samples from 63 patients. In each run, up to 16 paired serum and plasma samples from eight patients on 316v2 were processed. Run median output was 257 Mb (Mega bases), median read length was 124bp, mean read depth was 2821x and coverage uniformity was 97%. When the 63 samples were tested with the SiRe panel, the cfDNA of all eight patients with wild-type tumour tissue was negative (specificity 100%, CI 64.6-100%). In the remaining

55 patients with EGFR, KRAS, NRAS or BRAF mutations in tumour tissue, the SiRe panel detected the same mutation in the serum and/or plasma cfDNA in 46 cases (sensitivity 83.6%, CI 67.3–94.3%;). (Table 5.)

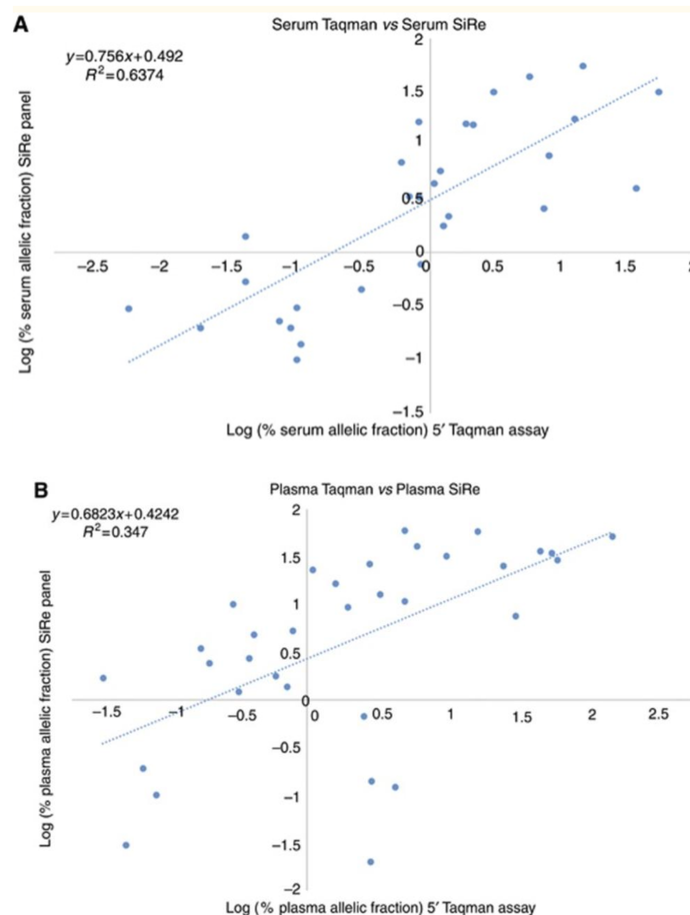
	TDA (cfDNA)		
SiRepanel (cfDNA)	**Mut +**	**Mut -**	**Total**
Mut +	42	4	46
Mut -	0	17	17
Total	42	21	63

Table 5.Concordance of Taqman-derived assay (TDA) and the SiRe panel NGS in retrospective serum and plasma cfDNA samples.

Comparison of the SiRe panel with a TDA in cfDNA samples.

We compared the performance of the SiRe panel for mutation analysis in cfDNA with that of a previously reported TDA (28, 29) in 63 samples: (i) the 40 cfDNA samples obtained at presentation mentioned above; (ii) archival serum and plasma cfDNAs from 12 patients in response to different types of antitumor drugs; and 11 patients mutations in the cfDNA of 46 of 63 patients. The test was positive in both serum and plasma cfDNA in 35 patients (76.1%), positive in plasma but not in serum in 5 patients (10.9%), and positive in serum but not in plasma in 6 patients (13%). An EGFR sensitising mutation and the p.T790M resistance mutation were detected simultaneously in 10 patients at progression to EGFR TKIs. As reported in Table 5, there was a high concordance (Cohen's Kappa 0.85) between the results obtained with the NGS SiRe panel and the TDA, although the performance of the SiRe was slightly better. All 42 patients with mutation-positive cfDNA at TDA were also positive with

the SiRe panel, and the 17 negative samples with the panel were also negative at TDA. In addition, NGS detected mutations in the cfDNA of four patients, whereas TDA did not. The mutations in these four patients appeared also in paired tumour tissue. One was a p.L597R mutation in BRAF not covered by the TDA, and was confirmed by dPCR. The remaining three mutations were a p.L861Q mutation in EGFR and two KRAS mutations, p.G12C and p.G12A. Both TDA and NGS using the SiRe panel enable quantification of the mutated alleles. There was a significant correlation in the levels of serum cfDNA between the two techniques (r=0.64). In contrast, correlation was lower in the case of plasma (r=0.35), but improved significantly when three outlier samples were removed (r=0.61). (Figure 3.)



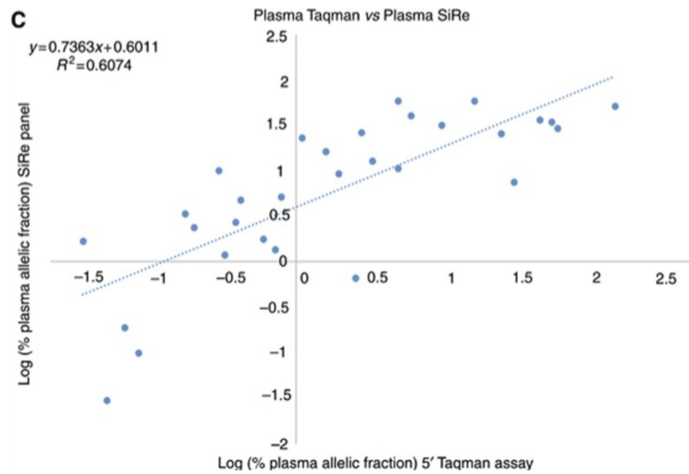


Figure 3. Quantification of mutated allele fractions. Comparison of the quantification of mutated allele fractions by Taqman Derived Assay *vs* SiRe NGS in serum (**A**) and plasma (**B**) cfDNA. In the case of plasma, three outliers were removed and results re-plotted (**C**). (Ref. 17)

Evaluation of the SiRe panel for prospective analysis of clinical samples.

The performance of the SiRe panel in the clinical setting was evaluated by prospectively testing the serum and plasma cfDNA of patients with advanced NSCLC for whom no tissue was available in order to select them for TKI treatment. Seventy-nine patients were tested, 46 at presentation and 33 at the time of tumour progression after first-line TKI treatment (Table 4). The NGS procedure was adequate for variant calling in the 79 cfDNA paired serum and plasma samples. The run metrics parameters were not dissimilar from those of the retrospective samples. In fact, in prospective cfDNA samples, the median output was 210Mbases, the median read length 125.57bp, the mean read depth 3385.45 and coverage uniformity 97.49%. Among the 46 patients analysed at baseline, we detected four EGFR mutations (8.7%), one point mutation in exon 18 (p.G719A), two deletions in exon 19 (both p.E746_A750delELREA) and one insertion in exon 20 (p.H773-V774insH). In all four patients, the mutant alleles were detected in both serum and plasma cfDNA and were confirmed by digital PCR (data not

shown). Regarding samples at progression, the SiRe panel did not detect mutations in 12 patients, whose tissues had been identified as EGFR wild type in biopsies at presentation. In contrast, among the 21 patients EGFR positive in baseline tissue, the SiRe panel confirmed the same mutation in cfDNA in 19 cases (Table 6).

	At presentation			**At response or progression**		
	TDA+ Sanger (FFPE tumour tissue)			**TDA+ Sanger (FFPE tumour tissue)**		
SiRe panel (cfDNA)	**Mut +**	**Mut -**	**Total**	**Mut +**	**Mut -**	**Total**
Mut +	28	0	28	18	0	18
Mut -	5	7	12	4	1	5
Total	33	7	40	22	1	23

Table 6. Comparison of the mutational status in FFPE tumor tissue at presentation with the results of the SiRe panel in archival cfDNA purified from serum and plasma baseline (n=42, left) and at response or after tumor progression (n=23, right)

Thus, sensitivity and specificity in this cohort of patients at progression were within the range of those observed in the retrospective cohort. Interestingly, in 9 of those 19 cases (47%), we observed the emergence of the EGFR p.T790M mutation in addition to the original EGFR activating mutation. The appearance of EGFR p.T790M mutation in relation to TKIs treatment regimen was reported in figure 4. Of the 28 mutations (sensitising +p.T790M) detected, 10 (35.70%) were present in both serum and plasma, 7 (25%) in plasma alone and 11 (39.3%) in serum alone. All mutations detected by the SiRe panel at progression were confirmed by dPCR.

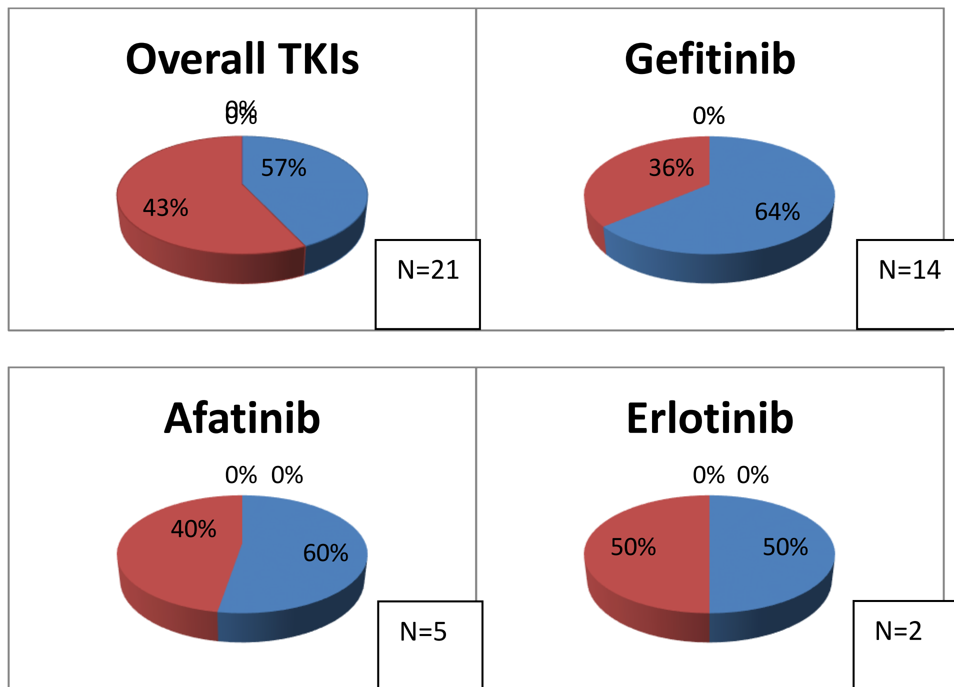


Figure 4. Frequency of the *EGFR* p.T790M mutation (green: T790M− red T790M+) after progression to thyrosine kinase inhibitors (TKIs) in the serum and plasma cfDNA of EGFR-mutated patients evaluated with SiRe panel NGS**.**

2.1.3 Discussion

In this chapter, we analyzed the performance of ultra-deep sequencing using a narrow NGS panel on Ion Torrent PGM is excellent, and how this procedure can be used for the routine testing of relevant tumour mutations in cfDNA. The high sensitivity (90.5%) and analytical specificity (100%) of this panel equal or even surpass those of such other procedures as real time PCR-based methods. Unlike earlier NGS applications that cover large genomic regions (27), our small gene panel (5.2kb) focuses on biomarkers that are currently used in the clinical setting. The ultra-deep sequencing procedure reported herein has various advantages. In fact, using a single panel, we were able to detect up to 568 relevant mutations in six genes (EGFR, KRAS, NRAS, BRAF, cKIT and PDGFRa). These mutations included less common, but actionable variants such as the BRAF p.L597R mutation in melanoma. Sequencing with the SiRe panel was more efficient than real-time PCR target techniques in detecting deletions (n=2) and point mutations (n=6) on cfDNA samples. In addition, NGS *per se* is a time-effective procedure for analysing large numbers of samples, thereby optimising the work flow in molecular pathology laboratories.(26) With our procedure, different types of samples (DNA from tumour tissues and cfDNAs from biological fluids) from patients affected by different types of diseases (e.g., NSCLC, GIST, CRC and melanoma) can be processed simultaneously. Consequently, sample batching is more effective and does not require a minimum number of a given tumour type. As a result, turnaround time (TAT) can be as short as three working days, as recommended by international guidelines.(31) The recently developed Ion Chef automated library preparation station, which has a better procedure reproducibility and standardisation than manual procedures, also contributes to the short TAT (26). The Ion Torrent PGM protocols, panels and variant caller do not detect low abundant mutations diluted in a large amount of WT DNA. Therefore, we used several in-house strategies specifically tailored to

cfDNA. Firstly, we reduced the number of genes and exons vs commercially available tests, and we modified the thresholds for variant calling, in particular all the variants with ≥5X allele coverage and a quality score ≥20, within an amplicon that covered at least 1000X alleles, were called. We also adapted the Ion Chef template preparation protocol by pooling two 16-sample libraries in each run. Thus, using this well standardized procedure, we were able to sequence simultaneously up to 32 paired plasma/serum samples in less than 3h on the PGM, with a consequent reduction in the total consumable cost. In a previous study (26) we showed that by using a commercially available 22 gene panel (AmpliSeq Colon and Lung Cancer Panel) on the Ion Torrent PGM, the consumable cost was euro 196 per sample. Using the modified protocol that we developed in this current study the cost per sample was lowered to 98 euro for simultaneously analysis of six different genes. This is comparable with the cost of the most commercially available Real Time PCR based kits. The simultaneous analysis of paired plasma/serum samples is a crucial feature of this new procedure since the sensitivity of somatic mutation analysis in cfDNA increases when serum and plasma are analysed together (28, 29). Our results are in agreement with this finding. In fact, of the 89 patients found to carry mutations in cfDNA, 58 (65.17%) were positive in both serum and plasma, 15 (16.85%) in plasma alone and 16 (17.98%) in serum alone. From the technical point of view, even when sequencing 16 samples simultaneously in a run, the SiRe panel had optimal run metrics in our daily clinical practice in terms of both mean depth reads and uniformity of coverage, which resulted in a high assay sensitivity in cfDNA vs tumour tissue (90.5%) and a specificity of 100%. This is a very high degree of concordance, particularly given the 91.7% concordance between paired surgical resection and cytological samples (32). Thanks to the high sensitivity of our assay, the EGFR mutational rate of 8.7% that we identified in NSCLC patients prospectively tested on cfDNA at baseline is in keeping with previous data on tissue samples. (33) Similarly, the frequency of the EGFR p.T790M mutation, which was detected in the cfDNA of 9 of 19 (47.4%) patients progressing after TKI treatment (n=5 gefitinib, n=3 afatinib,

n=1 erlotinib), is in line with data obtained on tissues samples collected after disease progression (29). The performance of our methodology compares favorably with that of NGS for mutational analysis in the blood of cancer patients. An Ion Torrent-derived sequencing of five genes in cfDNA purified from never smoking lung cancer patients achieved a modest 58% sensitivity and 87% specificity (34). An analysis of 23 amplicons in five genes using cfDNA from breast cancer patients identified 10 mutations but missed 6 identified by droplet digital PCR. (35) When restricted to EGFR, deep sequencing achieved 61–80% sensitivity and 94–98% specificity in advanced NSCLC.(36) The 90.5% sensitivity of our assay also exceeds the 77% recently reported when NSCLC plasma-derived cfDNA was analysed on an Illumina NGS platform with a panel covering amplicons of 11 clinically relevant genes.(37) Despite the variations inherent to the platforms used, such as the library preparation and the longer TAT (6 days), the Illumina-based NGS approach featured similar run metrics and analytical parameters as our assay, which supports the use of ultra-deep sequencing in the clinical setting.(37) It is conceivable that the higher sensitivity achieved by our panel is due not only to technical differences but also to the simultaneous testing of serum and plasma in each patient. Besides being an alternative to molecular diagnosis at presentation when tumour tissue is not available, liquid biopsy is also a non invasive test with which to monitor response to targeted therapy and to detect the emergence of resistance mutations in genes such as EGFR (38) and ESR1 (Chu et al, 2016). Monitoring would consist in quantifying the mutant allelic fractions in cfDNA over time, which can be reliably assessed by our NGS assay. The SiRe panel detects the appearance of resistance mutations such as EGFR p.T790M. Finally, the non-synonymous mutation burden correlates with a good response to immunotherapy in NSCLC (39) and other tumours, and NGS has been proposed as a tool with which to design customised immunotherapies that target common driver mutations.(40) Our panel, which covers several exons in frequently mutated genes, can be useful also in this setting. In conclusion, we have developed and translate in clinical setting an NGS assay based on a narrow gene panel. The assay detects relevant

mutations in cfDNA purified from the serum and plasma of patients with the tumours most commonly tested for molecular alterations (such as NSCLC, CRC and metastatic melanoma). The SiRe panel has excellent sensitivity and specificity, and is hence suitable for testing blood samples in the clinical setting. Finally, it enables the application of NGS on a prospective basis in daily molecular predictive pathology practice, particularly when tumour tissue is not available, and is a tool with which to monitor disease course.

Chapter 3

3.1.1 Cell free DNA analysis by SiRe® panel in a basal setting of NSCLC patients.

Non small cell lung cancer (NSCLC) is diagnosed in most cases at advanced stages of disease. Diagnostic samples are frequently scarcely cellular, being represented by either cytological specimens or small tissue endoscopic biopsies; these limited tissue samples often may be not sufficient for epidermal growth factor receptor (EGFR) and other clinical relevant biomarkers, such as ALK translocation and PD-L1 expression, whose assessment is required to select patients for first line treatment administration (40, 41). In particular for EGFR tyrosine kinase inhibitors (TKIs), such as gefitinib, erlotinib and afatinib the identification of activating EGFR mutations in exon 18, 19 and 21 is mandatory before the first line treatment (33, 42- 46). To date according to the European Medicines Agency guidelines, in patients without tissue availability, only for EGFR TKIs treatment decision making, cell-free DNA (cfDNA) can be used as a fast and non-invasive surrogate for EGFR mutational testing (17, 29, 47- 49). However, the assessment of gene mutations in cfDNA is challenging, in particular in basal setting, for the detection of first and second TKIs generation EGFR sensitizing mutations, due to the very low concentration of circulating tumor DNA, that represent only a small fraction of the total cfDNA. (17,48,49,50-52) Thus, the clinical implementation of next generation techniques, such as next generation sequencing (NGS) or digital PCR (dPCR) based assay is crucial (17,48,49,50,53,54,). In a recent study our laboratory validated the SiRe® NGS panel technical performance for

mutation detection in EGFR, KRAS, NRAS, BRAF, cKIT and PDGFR starting from cfDNA retrieved from patients with different solid tumors (NSCLC, metastatic colo-rectal cancer, melanoma and gastrointestinal stromal tumor).(17) SiRe® showed a lower limit of detection (0.01%) and an higher analytical performance respect to a very sensitive modified TaqMan probe real time PCR based approach.(17) The analysis of cfDNA gene mutations was carried out using as gold standard the mutational status obtained on matched tissue derived DNA, but little is known regarding the application of this approach in clinical setting, in particular in baseline setting of NSCLC patients, prior to EGFR TKIs administration, without a referent DNA derived tissue to confirm the mutational data obtained on cfDNA.(55) The aim of the present study was to review the NGS data obtained by using SiRe® NGS panel starting from cfDNA collected in routine NSCLC baseline setting to prospectively select patients, without tissue availability, for first and second generation EGFR TKIs treatment administration.

3.1.2 Material and Methods

From January 2017 to March 2017 , n=64 liquid biopsy analysis was requested from the oncologists of different South Italy institutions (n=14), following the European Medicines Agency guidelines, for the analysis of EGFR mutations on cfDNA in NSCLC patients without tissues availability at presentation, to assess the eligibility to first and second generation EGFR TKIs (Table 7). On the overall n=39 men and n=25 women were analyzed with a mean age of 66 years (range, 36–89 years). For each patient, 10 mL of blood was collected in-house by using EDTA Vacutainer tubes (BD, Plymount, UK) by a dedicated nurse at the Department of Public Health

of the University of Naples Federico II. The protocols adopted in this study were previously validated (13). Briefly, before cfDNA extraction, two centrifugation steps (2,300 rpm for 10 min) were carried out to obtain at least 1.2 mL of plasma for each patient. cfDNA was extracted by using the QIAsymphonyDSPVirus/Pathogen Midi Kit on the QIAsymphony robot (Qiagen, Venlo Limburg) accordingly with the manufacturer instructions. By using SiRe panel, following the previously validated protocol, libraries were automated constructed and purified using Ion AmpliSeq DL8 Kit (Thermofisher) on the Ion Chef instrument (Thermofisher) and, after barcoding, purified libraries derived from eight cfDNA plasma samples were diluted and combined with eight additional cfDNA-derived libraries to obtain a 16 Ion Code pooled library, re-loaded into the Ion Chef instrument for template preparation by using the Ion PGM Hi-Q IC Kit (Thermofisher). Finally, templates were loaded into the 316v2 chip and sequenced on Personal Genome Machine (PGM). Signal processing and base calling were carried out using the default base caller parameters on Torrent Suite (v.5.0.2) and coverage analysis was performed using SiRe specific bed files with coverage plug-in (v.5.0.2.0). In addition to automatic variant calling analysis, by using SiRe panel specific optimized variant caller plug-in (v.5.0.2.1) parameters, BAM files were visually inspected with the Golden Helix Genome Browser v.2.0.7 (Bozeman, MT, USA). Only variants with >5× allele coverage and a quality score >20, within an amplicon coverage at least 1,000× alleles, were reported and the relative mutated allele frequency was annotated, considering not only EGFR, but also KRAS, BRAF and NRAS gene hotspots region, relevant for NSCLC and covered by the SiRe panel. Written informed consent was obtained from all patients and documented 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) and all samples were handled in compliance with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/).

3.1.3 Results

The SiRe NGS analysis results were adequate in 98.4% of cases (63/64) accordingly to the quality parameters reported in the methods section and previously validated; only one cases (#30) failed to reach the quality thresholds for data analysis. Regarding the run metrics parameters (Table 7), the median number of reads for sample was 120,960, the median number of read length was 127 bp, the median number of mapped reads was 120,498, the mean percentage of reads on target was 97%, the average reads for amplicon was 2,894 and the uniformity of coverage was 98%, in accordance with the data obtained in our previous validation study (17). On the overall, considering EGFR, KRAS, NRAS and BRAF genes, 24 patients (38%) showed at least one mutation. Only one patient (#7) showed two concomitant mutations (NRAS p.G13D and KRAS p.Q61H). In particular, 5 EGFR mutations (8%) were detected [n=2, exon 19 deletions (both p.E746_A750delELREA); n=2, exon 20 insertions (p.H773_ V774insH and V769_D770insASV); and n=1, p.L858R exon 21 point mutation]; 14 KRAS point mutations (22%) [n=11, exon 2 mutations (n=4 p.G12C, n=3 p.G12D, n=1 p.G12S, n=1 p.G13D and n=2 p.G13S); and n=3, exon 3 point mutations (n=1 p.A59V and n=2 p.Q61H)]; n=4 NRAS point mutations (6%) [n=2, exon 2 mutations (n=1 p.G12Sand n=1 p.G13D); and n=2, exon 3 point mutations (n=1 p.A59C and n=1 Q61P)]; 2 (3%) BRAF point mutations [n=1 exon 11 p.G469A mutation and n=1 exon 15 p.V600E mutation]. The mutated allele frequency for each mutation detected is reported in Table 1. Prior to clinical reporting,

only the EGFR detected mutations by the SiRe panel were also confirmed by digital PCR based assay. An example of this approach was showed in Figure 5.

Patients	Sex	Age	Reads	Mean Read Length	Number of mappedreads	% reads on target (%)	Averagereads per amplicon	Uniformity of ampliconcoverage (%)	NRAS (allelicfrequency)	BRAF (allelicfrequency)	EGFR (allelicfrequency)	KRAS (allelicfrequency)
1	M	76	325,098	127	324,684	97.31	7,523	100.00	WT	WT	WT	WT
2	F	77	223,871	130	223,209	97.71	5,187	100.00	WT	WT	WT	WT
3	M	68	288,543	127	287,41	97.60	6,679	100.00	WT	WT	WT	WT
4	F	74	26,017	125	25,666	96.70	590.9	97.62	G12S (1.00%)	WT	WT	WT
5	F	36	163,218	128	162,752	98.06	3,8	97.62	WT	WT	WT	G12D (1.50%)
6	F	50	146,656	127	145,906	97.25	3,378	100.00	WT	V600A (0.20%)	WT	WT
7	M	70	173,021	127	172,314	97.55	4,002	97.62	G13D (0.34%)	WT	WT	Q61H (0.20%)
8	M	64	151,445	127	150,37	97.03	3,474	97.62	WT	WT	WT	G12C (1.30%)
9	M	61	75,353	127	74,972	97.68	1,744	97.62	WT	WT	WT	WT
10	M	45	190,62	126	189,708	97.49	4,403	100	WT	WT	H773_V774insH (37.0%)	WT
11	M	48	103,023	128	102,573	97.79	2,388	97.62	WT	WT	WT	G12C (0.60%)
12	F	61	163,363	128	162,841	97.75	3,79	97.62	WT	WT	WT	WT
13	F	57	130,596	128	130,25	97.72	3,031	97.62	WT	WT	WT	WT
14	F	51	155,551	128	155,018	97.40	3,595	97.62	WT	WT	WT	WT
15	M	62	60,927	128	60,611	97.77	1,411	97.62	WT	WT	V769_D770insASV (12.30%)	WT
16	F	87	67,571	128	67,404	97.85	1,57	97.62	WT	WT	WT	WT
17	F	77	181,295	129	180,633	98.06	4,217	95.24	WT	WT	WT	WT
18	M	75	104,653	127	104,925	97.79	2,429	97.62	WT	WT	WT	WT
19	M	58	262,771	130	261,202	97.94	6,091	97.62	WT	WT	WT	WT
20	F	38	248,897	128	248,287	97.68	5,775	97.62	WT	WT	WT	WT
21	F	51	169,989	128	169,581	97.44	9,734	97.62	WT	WT	WT	WT
22	M	64	36,774	128	36,693	97.84	854.7	97.62	WT	WT	WT	WT
23	M	74	195,245	128	194,624	97.50	4,518	100.00	WT	WT	L858R (3.20%)	WT
24	F	54	82,48	128	82,303	97.80	1,916	97.62	WT	WT	WT	WT
25	M	84	12,499	127	12,439	97.79	289.6	97.62	WT	WT	WT	WT
26	F	59	71,556	129	71,36	97.54	1,657	97.62	WT	WT	WT	G12D (1.30%)
27	M	68	62,909	128	62,723	97.61	1,458	97.62	WT	WT	WT	WT
28	M	56	51,993	128	51,453	97.44	1,194	100.00	WT	WT	WT	WT
29	M	89	21,306	127	21,126	97.12	488.5	97.62	WT	WT	WT	G12D
30	M	53	76	60	66	39.39	0.69	91.45	Failed	Failed	Failed	Failed
31	F	67	49,539	129	49,435	97.50	1,148	97.62	WT	G469A (5.00%)	WT	WT
32	M	62	113,202	130	112,93	98.01	2,635	97.62	WT	WT	WT	G12C (5.63%)
33	M	70	72,936	129	72,752	97.77	1,694	97.62	WT	WT	WT	WT
34	F	71	133,127	129	132,803	97.93	3,096	97.62	WT	WT	WT	WT

35	F	75	78,508	128	78,34	97.55	1,82	97.62	WT	WT	WT	WT
36	M	80	175,862	130	175,177	97.66	4,073	97.62	WT	WT	WT	WT
37	M	66	118,804	129	118,575	97.87	2,763	97.62	WT	WT	WT	WT
38	F	71	139,472	129	139,008	97.23	3,218	100.00	WT	WT	WT	WT
39	M	67	111,5	128	110,909	97.27	2,571	97.62	WT	WT	WT	WT
40	M	64	348,181	135	347,148	97.91	8,093	97.62	WT	WT	WT	G13S (0.20%)
41	M	80	131,59	129	131,257	97.98	3,062	97.62	WT	WT	WT	WT
42	M	80	141,018	129	140,564	98.15	3,285	97.62	Q61P (0.14%)	WT	WT	WT
43	F	76	72,635	128	72,364	97.77	1,685	97.62	WT	WT	WT	G13D (0.30%)
44	M	42	152,707	127	152,031	97.58	3,532	97.62	A59C (0.20%)	WT	WT	WT
45	M	66	74,945	127	74,63	97.64	1,735	97.62	WT	WT	WT	WT
46	F	57	50,945	128	50,607	97.58	1,176	97.62	WT	WT	WT	WT
47	M	67	84,001	130	83,802	97.69	1,949	97.62	WT	WT	WT	WT
48	M	69	133,275	132	132,908	97.57	3,087	97.62	WT	WT	WT	G12S (6.40%)
49	M	65	92,859	126	91,962	96.90	2,122	97.62	WT	WT	WT	G12C (3.30%)
50	M	77	47,845	130	47,653	97.32	1,104	97.62	WT	WT	WT	Q61H (4.50%)
51	M	82	23,283	129	23,118	97.44	5,363	97.62	WT	WT	WT	WT
52	F	77	31,713	129	31,599	97.86	9,363	97.62	WT	WT	WT	WT
53	M	84	121,037	129	120,379	97.44	2,793	97.62	WT	WT	WT	G13S (0.20%)
54	F	73	73,937	129	73,23	97.42	1,699	97.62	WT	WT	WT	WT
55	M	66	136,734	131	136,185	97.37	3,157	97.62	WT	WT	WT	WT
56	M	69	102,126	130	101,718	97.30	2,356	97.62	WT	WT	WT	WT
57	M	65	103,211	131	102,957	97.48	2,39	97.62	WT	WT	WT	WT
58	M	65	73,517	129	73,31	97.32	1,699	97.62	WT	WT	WT	WT
59	M	81	163,403	130	162,818	97.33	3,773	97.62	WT	WT	WT	WT
60	M	68	182,161	130	181,087	97.19	4,19	97.62	WT	WT	WT	WT
61	F	54	64,662	127	63,955	97.52	1,485	97.62	WT	WT	WT	WT
62	F	64	119,32	130	118,669	97.21	2,747	97.62	WT	WT	WT	A59V (0.20%)
63	F	67	103,023	128	102,573	97.79	2,388	97.62	WT	WT	ELREA (5.40%)	WT
64	F	61	173,021	127	172,314	97.55	4,002	97.62	WT	WT	ELREA (0.70%)	WT

Table 7. Patients characteristics, SiRe next generation sequencing (NGS) panel run metric parameters (reads, mean read length in base pair, number of mapped reads, percentage of read on target, average reads per amplicon, uniformity of amplicon coverage) and genes mutational status with relative mutated allele frequency are reported for each sample.

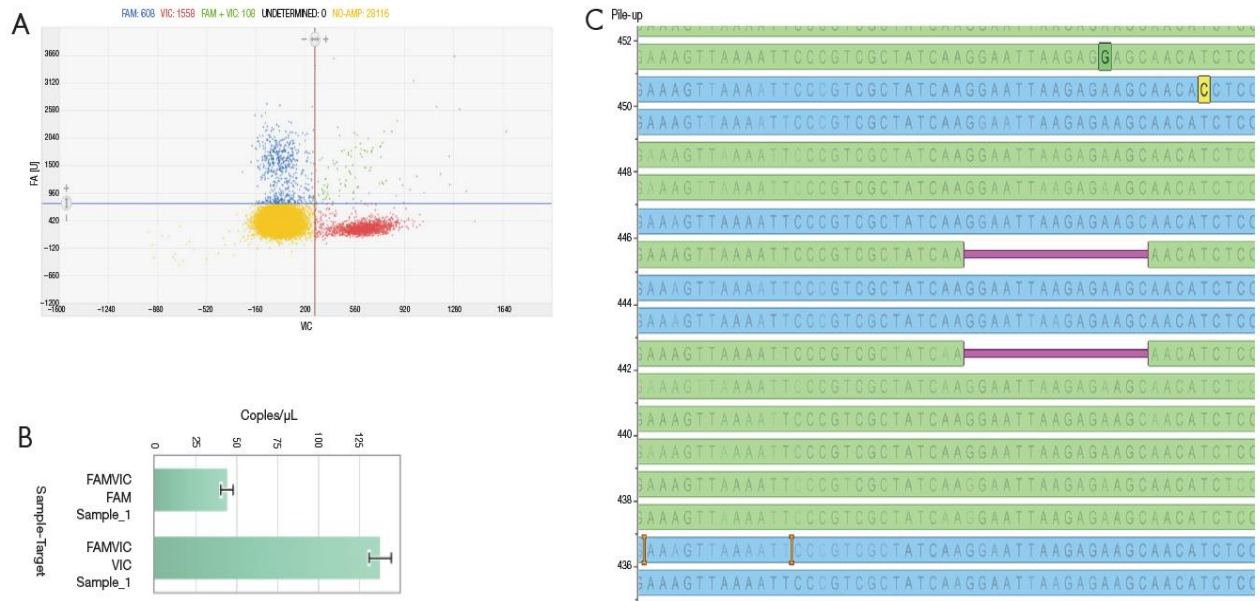


Figure 5. Case n.64 is reported. Digital PCR Quant Studio 3D cloud software (Thermofisher) was used to analyze the scatter plot (A) and the copies of mutated and wild type alleles detected in one µl of the extracted cell-free DNA (cfDNA) (B). In the panel (C), the SiRe panel next generation sequencing (NGS) result is reported obtained on the same extracted cfDNA and analyzed by using Golden Helix Genome Browser v.2.0.7 (Bozeman, MT, USA) and showing an epidermal growth factor receptor (EGFR) exon 19 deletion (p.E746_ A750delELREA).

3.1.4 Discussion

Data, generated by the SiRe NGS panel on cfDNA, prospectively collected from NSCLC patients, without tissue availability, examined for first and second generation EGFR TKIs treatment administration, are here reported; the performance of this NGS panel designed to cover only the current clinical relevant mutations, was more than excellent. Our data confirm previous validation data. Preliminary, we had prospectively analyzed a total of 79 NSCLC patients on cfDNA. In 46 instances, cfDNA had been derived from NSCLC patients at presentation; in this subset, we detected four EGFR mutations (8.7%); more in details, these were one point mutation in exon 18 (p.G719A), two deletions in exon 19 (both p.E746_A750delELREA) and one insertion in exon 20 (p.H773-V774insH) (17). Here, in this current subsequent study, we detected two exon 19 deletions (both p.E746_A750delELREA), two exon 20 insertions (p.H773_ V774insH; V769_D770insASV) and one p.L858R exon 21 point mutation. Thus, we confirm an overall EGFR mutation rate of 8.0%. In all instances, the EGFR mutations were always confirmed by an independent orthogonal dPCR based assay (Figure 5). In addition, in the present study we have also sequenced, in the same sample set, KRAS, NRAS and BRAF NSCLC relevant hot-spot regions, reporting an overall mutation rate of 38%. In particular, we detected 22% KRAS, 6% NRAS and 3% BRAF mutated samples, with only one patient that showed two concurrent mutations (NRAS p.G13D and KRAS p.Q61H). It is remarkable to note that the mutation distribution in cfDNA of this NSCLC baseline patient series was very similar to that reported on tissues derived DNA by previous studies exploiting a multi-gene assay in NSCLC (55-58). As a general rule, in the clinical trial settings the analysis of cfDNA had as a reference the mutational status obtained on tissue derived DNA (55-58). Conversely, following the European Medicines Agency guidelines, in baseline setting, the cfDNA analysis is indicated only for those patients in which tissues is not available. For this reason, the ability of SiRe®, to detect also mutation in KRAS,

NRAS and BRAF genes, offer an internal control in patients that do not show alterations in EGFR, considering that in the most part of the cases these mutations in these genes are mutually exclusive. In conclusion, our data update and confirm that SiRe NGS panel represents a robust analytical tool for a centralized laboratory enabling the possibility to test cfDNA mutational status in basal setting of NSCLC patients when no tissue samples are available to assess EGFR mutational status for first line treatment decision making.

References

1. Ahmadzada T, Kao S, Reid G, et al. An Update on Predictive Biomarkers for Treatment Selection in Non-Small Cell Lung Cancer J Clin Med. 2018 Jun; 7: 153.
2. Cree I, Deans Z, Marjolijn J et al. Guidance for laboratories performing molecular pathology for cancer patients J ClinPathol. 2014 Nov; 67: 923–931.
3. Elizabeth McPherson Genetic Diagnosis and Testing in Clinical Practice Clin Med Res. 2006 Jun; 4: 123–129.
4. Zhang R, Wenjun Yang, Zhang R, Yang W et al . Circulating Tumor DNA as a Liquid Biopsy in Cancer. ClinOncol. 2017; 2: 1265.
5. Robertson DJ, Lee JK, Boland CR et al.Recommendations on Fecal Immunochemical Testing to Screen for Colorectal Neoplasia: A Consensus Statement by the US Multi-Society Task Force on Colorectal Cancer. Gastroenterology. 2017;152:1217-1237.
6. GISCOR Survey CCR 2014 (www.giscor.it)
7. Bubendorf L, Büttner R, Al-Dayel Fet al. Testing for ROS1 in non-small cell lung cancer: a review with recommendations Virchows Arch. 2016; 469: 489–503.
8. Treece A, Montgomery N,Patel Net al. Fine-Needle Aspiration Smears as a Potential Source of DNA for Targeted Next-generation Sequencing of Lung Adenocarcinomas Cancer Cytopathol. 2016 Jun; 124: 406–414.
9. Volik S, Alcaide M, Morin Ret al. Cell-free DNA (cfDNA): Clinical Significance and Utility in Cancer Shaped By Emerging Technologies Mol Cancer Res. 2016 Oct;14: 898-908.
10. Rolfo C, Mack P, Scagliotti GV et al. Liquid Biopsy for Advanced Non-Small Cell Lung Cancer (NSCLC): A Statement Paper from the IASLC. J ThoracOncol. 2018 Sep;13:12481268.

11. Sepulveda AR, Hamilton SR, Allegra CJ et al. Molecular Biomarkers for the Evaluation of Colorectal Cancer: Guideline From the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology. Arch Pathol Lab Med. 2017; 141:625-657.
12. Goldberg RM, Montagut C, Wainberg ZA et al. Optimising the use of cetuximab in the continuum of care for patients with metastatic colorectal cancer. ESMO Open. 2018 5; 3:e000353.
13. Imperiale TF, Ransohoff DF, Itzkowitz SH et al.Multitarget stool DNA testing for colorectal-cancer screening. N Engl J Med. 2014 10;371: 187-8.
14. Nian J, Sun X, Ming S et al. Diagnostic Accuracy of Methylated SEPT9 for Blood-based Colorectal Cancer Detection: A Systematic Review and Meta-Analysis. ClinTranslGastroenterol. 2017;8: e216.
15. Schneider M, Scholtka B, Gottschalk U et al.Detection of up to 65% of Precancerous Lesions of the Human Colon and Rectum by Mutation Analysis of APC, K-Ras, B-Raf and CTNNB1. Cancers (Basel). 2010 29;3: 91-105.
16. Scholtka B, Schneider M, Melcher R et al. A gene marker panel covering the Wnt and the Ras-Raf-MEK-MAPK signalling pathways allows to detect gene mutations in 80% of early (UICC I) colon cancer stages in humans. Cancer Epidemiol. 2009; 33: 123-9.
17. . Malapelle U, Mayo de-Las-Casas C, Rocco D et al. Development of a gene panel for next-generation sequencing of clinically relevant mutations in cell-free DNA from cancer patients. Br J Cancer. 2017;116: 802-810.
18. Anosova I, Kowal EA, Dunn MR et al.. The structural diversity of artificial genetic polymers. Nucleic Acids Res. 2016 18;44:1007-21.
19. Powell M, Peletskaya E, Sun Q,et al.Development and validation of ColoScape , a new colorectal cancer mutation detection assay, 2018, J ClinOncol 36: suppl; abstr e24189.
20. Papadopoulos N, Kinzler KW, VogelsteinBet al. The role of companion diagnostics in the

development and use of mutation-targeted cancertherapies. Nat Biotechnol,2006, 24: 985–995.

21. Di Nicolantonio F, Martini M, Molinari F et al. Wild type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. J ClinOncol, 2008; 26: 5705–5712.
22. Lievre A, Bachet JB, Boige V et al. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. J ClinOncol, 2008, 26:374–379.
23. Lynch TJ, Bell DW, Sordella R, et al Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med, 2004, 350: 2129–2139.
24. Chapman PB, Hauschild A, Robert C. et al. BRIM-3 Study Group. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. NEngl J Med 2011, 364: 2507–2516.
25. Antonescu CR, Targeted therapies in gastrointestinal stromal tumors. SeminDiagnPathol, 2008, 25: 295–303.
26. Malapelle U, Pisapia P, Sgariglia R et al. Less frequently mutated genes in colorectal cancer: evidences from next-generation sequencing of653 routine cases. J ClinPathol, 2016, 69: 767–771.
27. Cancer Genome Atlas Research Network. Comprehensive molecular profiling of lung adenocarcinoma. Nature 2014, 511: 543–550.
28. Gonzalez-Cao M, Mayo-de-Las-Casas C, Molina-Vila MA et al. BRAF mutation analysis in circulating free tumor DNA of melanoma patients treated with BRAF inhibitors. Melanoma Res, 2015, 25: 486–495.
29. Karachaliou N, Mayo-de las Casas C, Queralt C et al. Association of EGFR L858R mutation in circulating free DNA with survival in the EURTAC Trial. JAMA Oncol, 2015,

1:149–157.

30. Malapelle U, de Luca C, Vigliar E et al. EGFR mutation detection on routine cytological smears of non-small cell lung cancer by digital PCR: a validation study.J ClinPathol, 2016, 69: 454–457.
31. Lindeman NI, Cagle PT, Beasley MB et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. J MolDiagn, 2015, 15: 415–453.
32. Sun PL, Jin Y, Kim H, Lee CT et al. High concordance of EGFR mutation status between histologic and corresponding cytologic specimens of lung adenocarcinomas. CancerCytopathol, 2013, 121: 311–319.
33. Malapelle U, Bellevicine C, De Luca C et al, EGFR mutations detected on cytology samples by a centralized laboratory reliably predict response to gefitinib in non small cell lung cancer patients. CancerCytopathol, 2013, 121: 552–560.
34. Couraud S, Vaca-Paniagua F, Villar S, et al. Noninvasive diagnosis of actionable mutations by deep sequencing of circulating free DNA in lung cancer from never smokers: a proof-of- concept study from BioCAST/IFCT-1002. Clin CancerRes 2014,20: 4613–4624.
35. Guttery DS, Page K, Hills A et al. Noninvasive detection of activating estrogen receptor 1 (ESR1)mutations in estrogen receptor positive metastatic breast cancer. ClinChem, 2015, 61: 974–982.
36. Uchida J, Kato K, Kukita F et al. Diagnostic accuracy of noninvasive genotyping of EGFR in lung cancer patients by deep sequencing of plasma cell-free DNA. ClinChem, 2015, 61: 1191–1196.
37. Paweletz CP, Sacher AG, Raymond CK et al. Bias-corrected targeted next-generation sequencing for rapid, multiplexed detection of actionable alterations in cell-free DNA

from advanced lung cancer patients. Clin Cancer Res, 2016, 22: 915–922.

38. Sundaresan TK, Sequist LV, Heymach JV et al. Detection of T790M, the acquired resistance EGFR mutation, by tumor biopsy versus noninvasive blood-based analyses. Clin Cancer Res, 2016, 22: 1103–1110.
39. Rizvi NA, Hellmann MD, Snyder A et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1blockade in non-small cell lung cancer. Science, 2016, 348: 124–128.
40. Nielsen JS, Sedgwick CG, Shahid A et al. Toward personalized lymphoma immunotherapy: identification of common driver mutations recognized by patient CD8þ T cells. Clin Cancer Res, 2016, 22: 2226–2236.
41. Ofiara LM, Navasakulpong A, Ezer N, et al. The importance of a satisfactory biopsy for the diagnosis of lung cancer in the era of personalized treatment. CurrOncol 2012, 19:S16-23.
42. Wang S, Yu B, Ng CC, et al. The suitability of small biopsy and cytology specimens for EGFR and other mutation testing in non-small cell lung cancer. Transl Lung Cancer Res 2015, 4:119-25.
43. Chan BA, Hughes BG et al. Targeted therapy for non-small cell lung cancer: current standards and the promise of the future. Transl Lung Cancer Res, 2015, 4:36-54.
44. Köhler J, Schuler M et al. Afatinib, erlotinib and gefitinib in the first-line therapy of EGFR mutation-positive lung adenocarcinoma: a review. Onkologie 2013, 36:510-8.
45. Rosell R, Carcereny E, Gervais R, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomized phase 3 trial. Lancet Oncol 2012, 13:239-46.
46. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009, 361:947-57.

47. Wu YL, Zhou C, Hu CP, et al. Afatinib versus cisplatin plus gemcitabine for first-line treatment of Asian patients with advanced non-small-cell lung cancer harbouring EGFR mutations (LUX-Lung 6): an open-label, randomised phase 3 trial. Lancet Oncol 2014, 15:213-22.

48. Crowley E, Di Nicolantonio F, Loupakis F, et al. Liquid biopsy: monitoring cancer-genetics in the blood. Nat Rev ClinOncol 2013, 10:472-84.

49. Schwarzenbach H, Hoon DS, Pantel Ket al. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011, 11:426-37.

50. Malapelle U, Pisapia P, Rocco D, et al. Next generation sequencing techniques in liquid biopsy: focus on non small cell lung cancer patients. Transl Lung Cancer Res 2016, 5:505-10.

51. Qin Z, Ljubimov VA, Zhou C, et al. Cell-free circulating tumor DNA in cancer. Chin J Cancer 2016, 35:36.

52. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. SciTransl Med 2014, 6:224ra24.

53. Bennett CW, Berchem G, Kim YJ, et al. Cell-free DNA and next-generation sequencing in the service of personalized medicine for lung cancer. Oncotarget 2016, 7:71013-35.

54. Vendrell JA, Mau-Them FT, Béganton B, et al. Circulating Cell Free Tumor DNA Detection as a Routine Tool for Lung Cancer Patient Management. Int J MolSci 2017, 18. pii: E264.

55. Jänne PA, Yang JC, Kim DW, et al. AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. N Engl J Med 2015, 372:1689-99.

56. Goss G, Tsai CM, Shepherd FA, et al. Osimertinib for pretreated EGFRThr790Met-positive advanced non small-cell lung cancer (AURA2): a multicentre, open-label, single-arm, phase 2 study. Lancet Oncol 2016, 17:1643-52.

57. Mok TS, Wu YL, Ahn MJ, et al. Osimertinib or Platinum Pemetrexed in EGFR T790M-

Positive Lung Cancer. N Engl J Med 2017, 376:629-40.

58. Thress KS, Brant R, Carr TH, et al. EGFR mutation detection in ctDNA from NSCLC patient plasma: A cross platform comparison of leading technologies to support the clinical development of AZD9291. Lung Cancer 2015, 90:509-15.