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

"Molecular pathology in colorectal cancer: from early detection to treatment predictions."

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PON RICERCA E INNOVAZIONE

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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 aim of this thesis was to investigate the role of the molecular pathology in colorectal cancer from the "early detection" to treatment predictions. In particular, it has been investigated the role of the liquid biopsy as a screening tool for Colorectal Cancer (CRC) in comparison of Fetal Immunochemical Test (FIT) but also as a monitoring tool in patients with metastatic colorectal cancer to predict the possibility of relapse.

Moreover, in order to the treatment approach for CRC patients, it was evaluated the feasibility to perform the Idylla™ Assay to select the *wilde-type K-N-RAS* patients to the treatment with the monoclonal antibodies that target and inhibit the *EGFR* gene.

Methods: 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.

It was, also, performed the NGS analysis of the cfDNA samples isolated from mCRC patients and their matching metastatic tissue using the SiRe® panel, which covers 568 mutations in six genes (*EGFR, KRAS, NRAS, BRAF, cKIT* and *PDGFRα*) to monitor the possibility of relapse.

Moreover, it was assessed the *K-NRAS* mutational status by using a fully automated Real Time PCR, the Idylla™ System, as first-level screening method to quickly address the CRC patients to the best personalized treatment.

Results: 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%.

Regarding the NGS analysis of the cfDNA samples isolated from mCRC patients the molecular analysis was successfully performed in matched tissue specimen for a preliminary set (n=15) of enrolled patients. In n=3 cases, gDNA was not available for molecular analysis. In details, tissue sample analysis showed at least a molecular alteration in n=8/12 (66,6%) cases.

In order to evaluate the possibility to use the Idylla™ Assay to address the CRC patients to best target therapy n=450 mCRC patients and a total of 457 samples were tested. In particular, for 5 patients were analyzed also the metastatic tissue and for 2 patients we analyzed two synchronous cancers. A total of n= 200/457 (43,8%) samples gave a WT result, otherwise n=257/457 (56,2%) samples showed a mutation in the clinical relevant genes in patients with colorectal cancer.

In particular n=213/257 (82,9%) were KRAS positive, n= 20/257 (7,8%) were NRAS positive and n=24/257 (9,3%) were positive in BRAF gene.

Conclusions: In CRC patients setting, ColoScape is a promising tool for screening program aims to evaluate the triage of FIT+ patients and the cfDNA analysis, using the Sire® panel, has proven to be a valid monitoring tool to follow up the mCRC patients. Moreover, the Idylla ™ results combined with the treatment collected data from the oncologists could represent the best strategy to address *wild type* patients to a targeted therapy. The collected data obtained with this analysis could be useful to improve the treatment approach in the clinical practice setting.

Introduction

Colorectal cancer (CRC) is currently considered the fourth most common malignancy in the world, with an estimated 1.8 million new cases in 2018.[1] Risk factors for CRC development can be related to age (≥50), alcohol consumption, reduced physical activity, obesity, unbalanced diet, family history of intestinal polyps, and inflammatory diseases. It has been found that 95% of CRC cases are classifiable as adenocarcinomas. [2] In a not negligible percentage (20%) of CRC patients a metastatic disease (mCRC) was observed while high recurrence frequency (30-50%) was identified after surgical resection of primary tumor. [3] The median overall survival (OS) was estimated at 7.2-8 months for naïve CRC patients, but the emerging diagnostic and therapeutical approaches drastically improved OS (24-36 months).[4] These advances in the clinical administration of CRC patients were carried out thanks to innovative and revolutionary technical assays able to optimize diagnosis, prognosis, therapy and follow-up for CRC patients.[5] Nowadays, surgery represents the gold standard for the treatment of mCRC patients; however, an evaluable section of mCRC patients still relapse in 30-40% of cases.[6] In addition to this therapeutic option, since 2012 it was introduced in clinical practice the adoption of monoclonal antibodies (mAbs) against the epidermal growth factor receptor (*EGFR*) in stage IV patients with metastatic colorectal cancer that exhibited specific alterations in *KRAS, NRAS and BRAF* genes as negative predictive biomarkers for mCRC patients. [7] Molecular analysis of hot spot regions in the previously cited genes is crucial to evaluate the clinical response to mAbs (cetuximab and panitumumab), which have shown a clinical benefit for patients that do not exhibit hot spot mutations in predictive biomarkers genes.[8] Of note, *KRAS* mutations have been found in 40-45% of mCRCs and in 15-37% of early stages tumor patients. In addition, the concomitant *KRAS* and *BRAF* mutations, identified in a small section of clinical cases, decreases progression-free survival (PFS) and overall survival (OS) in mCRC patients. According to this aspect, the identification of several hot spot molecular alterations detected in different molecular biomarkers

is a driver for the better clinical administration of mCRC patients. Moreover, molecular qualification of detected alterations is pivotal for the evaluation of best therapeutic option. The most frequent mutation in *BRAF* (about 90% of cases), is detected in codon 600 of exon 15 (p.V600E) and is mutually exclusive with mutations in *RAS*. [9] In addition, studies have shown that metastatic patients that harbor *BRAF* mutation are characterized by a decrease in OS.[10] Although these emerging aspects plays a crucial role in the management of CRC patients, vast majority of advanced stage tumor patient relapse within 5 years. In this scenario, the identification in the early stage of malignant lesions is considered a new frontier in the tumor setting. [11] As regards, all colo-rectal adenomas can slowly evolve into malignant transformation. To demonstrate this, it has been observed that in patients with polyps with a diameter <10mm, no malignant transformation is found for at least 5 years. [12] It is important to consider, in addition to the characteristics of the polyp, the molecular features related to the mutational status of the precancerous lesion play a key role in molecular hallmark of malignant phenotype. In this regard, Vogelstein has extensively defined a series of molecular alterations associated with the increase of the malignancy index of the lesion (Figure 1). The identification of these alterations is crucial for the purpose of categorizing the malignancy status of the lesion.[13]

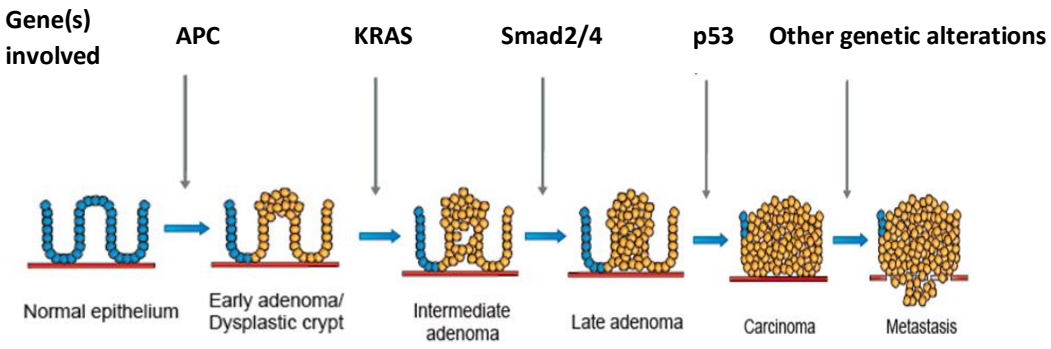

Fig. 1 - Vogelstein model for colorectal tumor genesis.

Regarding the evaluation of the molecular structure, two distinct pathways have been described that contribute to the development of overt neoplasia:

- the chromosomal instability pathway, in which the carcinoma originates from a conventional adenoma through a series of mutations in specific genes (*APC, KRAS, SMAD4* and *P53*);

- the serratus pathway, in which carcinoma develops towards the malignant form of sessile serratus adenoma through the development of mutations in the *BRAF* gene and by the accumulation of gene alterations resulting from the onset of the microsatellite instability (MSI-H) framework. [14]

In the clinical setting, the most widely used screening method for early screening of benign lesions of the colo-rectal tract is the so-called Fecal Immunochemical Test (FIT) based on the detection of fecal occult blood. However, this test is not very specific, since about 75% of FIT+ patients are negative at the next test performed by colonoscopy, which is the gold standard for the detection of pre-cancerous colorectal lesions.[15] In addition to the poor specificity shown by FIT, another issue concerns the technical (poor ability to store nucleic acids) and managerial (poor patient compliance) difficulties associated with the test (poor patient compliance) associated with the use of molecular methods for screening healthy patients.[16] Based on this evidence, it is appropriate to search for an alternative source from which to obtain nucleic acids for molecular testing, for the purpose of early diagnosis of pre-cancerous lesions, towards which patients show good compliance. For this reason, the use of blood sampling for the search of analytes suitable for this purpose is gaining ground. [17] Among the different molecules present in the circulatory stream, the one that can be best framed in this setting is circulating tumor DNA (ctDNA). The ctDNA may account for approximately 50% of the cell-free DNA (cfDNA) in patients in advanced states of metastasis, while only 1% in early stages of cancer (Fig. 2). [9] There are two ways in which ctDNA release into the circulation by tumor cells occurs and it can be attributed to two different hypotheses that complement each other:

- release through active mechanisms such as pro-metastasizing factors;

- release through passive mechanisms generally attributable to the high apoptotic index of tumor cells.[18]

Recently, techniques based on RT-qPCR approaches have been developed that allow to reach high levels of sensitivity, while having available very low ctDNA concentrations, for the search of mutations in target genes. [17] DiaCarta Inc, a Biotech company based in Richmond, California, has developed a high-sensitivity multiplex Real-Time PCR assay that combines a panel specific for genes typically mutated in CRC, according to the mutational model described by Vogelstein, with a proprietary technology that uses probes composed of xeno-nucleic acids (XNA) that block amplification of the wild-type allele. The presence of XNA allows DNA polymerase to be selective, amplifying mutated alleles while blocking wild-type alleles, maximizing analytical sensitivity. 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). 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.[19] 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.[20] 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.[21] 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.[22] The first chapter of this thesis concerns the development and validation of a novel diagnostic assay based on a target amplification of molecular alterations in key genes for the transition adenoma-carcinoma in CRC patients in order to consider liquid biopsy specimen as a promising screening tool in the management of FIT+ colorectal adenoma patients.

The second chapter will discuss the role of the liquid biopsy in the clinical monitoring of mCRC patients with proven liver metastases after surgical resection in order to predict the clinical outcome according to variation of molecular assessment by analyzing a serial blood withdrawn with ultra-deep NGS approach.

The aim of the third chapter of this thesis is to assess the combined performance of both CE-IVD approved Idylla™ *KRAS* and *NRAS* Mutation Tests, to comprehensively detect clinically relevant mutations to treatment eligibility with anti-*EGFR* of the colorectal cancer patients.

Chapter 1

1.1 Liquid biopsy based Coloscape™ 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).[23] Patients who test positive at FIT are referred to colonoscopy, where, however, about 75% of them turn out to be negative.[24] 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. [25] The assessment of wild-type status in the *RAS* gene is a prerequisite to the use of cetuximab and panitumumab, to give an example.[26] 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 [27] 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. [28] Thanks to the collaboration with DiaCarta Inc., a company based in Richmond (California), has been developed ColoScape™, an assay that combines a multiplex gene biomarker panel developed by Dr Bettina Scholka at the University of Postdam (Germany) [29-30] 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. To assess the Limit of Detection (LoD) of this Kit, and also the analytical parameters such as the sensitivity and the specificity, an analiytical valdation with cell line was performed. In this preliminary pilot study, the sensitivity and specificity of the ColoScape™ 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

Analytical validation

To assess the LoD (Limit of Detection of the ColoScape™ assay an analytical validation with cell line was performed. In detail, the genomic DNA was isolated from the cell lines *LS1034, C2BBE1, SW 1417, HDC 135, C2BBE1, HDC73, LS 1034, SW48, LS 174T, LIM 1215, C99, CW2, HCT 116, NC 14549, COLO 678.* The isolated genomic DNA was used by testing 1%, 0.5% and 0.1% mutant DNA template at 2.5ng, 5ng and 10 ng input for all the ColoScape™ targets *(APC, KRAS, CTNNB1* and *BRAF*).

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.[31] 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™assay test

The ColoScape™ 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).[29] 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™ 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.[32] XNA oligomers are not recognized by DNA-polymerases and cannot be utilized as primers in subsequent real-time PCR reactions. (Figure 2)

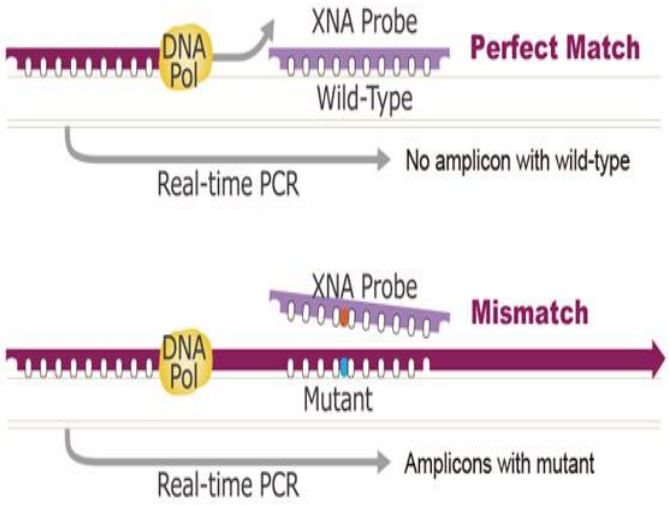

Fig. 2. The QClamp technology used by the ColoScape™ assay. XNA is a synthetic DNA analog that hybridizes tightly to complementary DNA target sequences only if the sequence is a complete match. When there is a mutation in the target site, and therefore a mismatch, the XNA-DNA duplexis unstable, allowing 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)	Cycles	Data Collection
Pre incubation	95	300	1.6	1	OFF
Denaturation	95	20	1.6		OFF
XNA Annealing	70	40	1.6	X50	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

Overall, 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 amount largely ranged from 0.4 to 9.0 ng/µL and, as expected, the extracted cfDNA amount from 10 mL plasma were higher than those ones from 5 mL plasma (median 2.9 vs 1.6 ng/µL). There were n=52 valid samples and n=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% (Table 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™ 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™ assay.

1.1.4 Discussion

Liquid-biopsy is a challenging type of sample for mutational analysis. We look for cf DNA and ct DNA. Estimates for ct DNA range from 1 to 10% of cf DNA. On top of this, mutations can occur at different allelic frequencies, which may be in some cases as low as 0.1%. [33] 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 Liquid biopsy as monitoring tool in the clinical managment of mCRC patients.

Colorectal Carcinoma represents the third most commonly diagnosed cancer in males and the second in females, with 1.8 million new cases and almost 861,000 deaths in 2018 according to the World Health Organization. [34] It was estimated over 1.3 million of new cases worldwide annually that are difficult to detect at the early stage for the absence of specific symptoms. [35] The principal metastasis site is the liver with a documented incidence of 40% in stage IIIB/IV of CRC patients. [35] Today tumor characterization depends on molecular signature evaluation that play a key role in the therapeutical setting especially. [36-37] From 2012 ASCO guidelines defined as mandatory the analysis of exon 2, 3 and 4 in Kirsten rat sarcoma (*KRAS*) and Neuroblastoma *RAS* Viral Oncogene Homolog (*NRAS*) genes in order to administrate a tailed therapy based on monoclonal antibodies (Cetuximab and Panitumumab) direct against Epidermal Grow Factor Receptor (*EGFR*). [38-41] Moreover the National Comprehensive Cancer Network (NCCN) guidelines (Version 2.2018) also recommended molecular assessment evaluation of v-Raf murine sarcoma viral oncogene homolog B (*BRAF*), to predict the prognostic degree of chemotherapeutic approach in mCRC patients.[42] In a considerable number of cases (~30%), small biopsy represents the only diagnostic available sample on which molecular test can be performed.[43] The high fragmentation profile derived from pre-analytical sample processing phases and the scant amount of nucleic acids generally recovered from the diagnostic sample highlights the inadequacy of conventional single-plex technology for the molecular analysis of increasing number of predictive biomarkers approved in the clinical practice for the clinical administration of lung cancer patients. In this scenario, the implementation of multiplex technology capable to successfully carry out molecular analysis of different biomarkers in diagnostic setting starting from a scant diagnostic material plays a pivotal role in the clinical stratification of diagnostic patients in clinical practice. As regards, Next Generation Sequencing (NGS) platforms

may be considered emerging diagnostic tools adopted in the clinical setting of solid tumor patients. Although several assays are commercially available to evaluate molecular assessment of diagnostic biomarkers, recent literature studies demonstrated that NGS panel may differentially respond to diagnostic query according to specific technical features. [44-46] Among them, the size of reference range, that consists in the escalating number of target biomarkers covered in the NGS analysis, still remains the most crucial aspect able to influence analytical performance of NGS analysis on "difficult" diagnostic specimens of lung cancer patients.

As regards, in our previous experience we have designed, developed and validated for both tissue samples and liquid biopsy specimens, a narrow NGS gene panel called SiRe® (in collaboration with Genedin s.r.l., Rome, Italy, a spin-off of the Department of Public Health, University of Naples "Federico II", Naples, Italy) that covers 568 clinical relevant mutations in seven genes (*EGFR*, *KRAS*, *NRAS*, *BRAF*, *cKIT*, *PDGFRa* and *PIK3CA*) involved in NSCLC, gastrointestinal stromal tumor (GIST), mCRC, melanoma and breast cancer patients (BC).[47-49] Despite of large diffusion of NGS platforms able to detect very low frequency clinically relevant mutations from scant diagnostic samples, a not negligible number of patients could not benefit of a tailored therapy due to inadequate molecular analysis performed on diagnostic available tissue specimen. In this scenario, liquid biopsy may represent an investigative and non-invasive approach that allows to easily recover circulating tumor DNA (ctDNA) that represents a small fraction of circulating free DNA (cfDNA) approved for diagnostic purposes in the management of lung cancer patients. Remarkably, liquid biopsy specimen is approved in the prediction of clinical response to TKIs for NSCLC patients at basal setting (when diagnostic available tissue specimen is not adequate to perform molecular analysis in NSCLC naïve patients) or resistance setting (for the identification of *EGFR* exon 20 p.T790M acquired resistance mutation after a first line of treatment with first or second generation TKI) Moreover, serial blood withdrawn may be easily performed on high compliant diagnostic patient; this aspect suggests a new application of this precious source of nucleic acids in clinical practice. In fact, several literature

studies evaluated the role of liquid biopsy for the monitoring of mutation allele frequencies (MAF) in key genes that play a crucial role in the clinical administration of solid tumor patients. [50,51]

Despite of new tailed molecular approach relative to therapy administration in mCRC patients, a critical point of question is represented by the high number of cases that relapse after surgical treatment. [52] In this field, cfDNA may be approached as a follow-up device able to estimate the minimal residual disease (MRD) for the treatment decision.[53] Tie *et al* validated a liquid biopsy-based workflow for the prediction of tumor recurrence in a cohort of stage II colon cancer patients.

At the sight of these data, liquid biopsy specimens are considered an emerging tool for this clinical application but the validation of a novel diagnostic approach should be also elucidated in order to optimize the clinical administration of mCRC patients after surgical resection.

This chapter will discuss the role of the liquid biopsy in the clinical monitoring of mCRC patients with proven liver metastases after surgical resection in order to predict the clinical outcome according to variation of molecular assessment by analyzing a serial blood withdrawn with ultra-deep NGS approach.

2.2 Material and methods

Study design

A total of n=30 liver metastatic mCRC patients will be prospectively enrolled at General Surgery affiliated to the Department of Public Health (University of Federico II, Naples). In details, n=4

1) 24h before liver metastasis surgical resection; 2) 48h post liver metastasis surgical resection; 3) 30 days post liver metastasis surgical resection; 4) 365 days post liver metastasis surgical resection serial evaluation timing point was collected for each patient. Moreover, corresponding tissue specimen after liver metastasis surgical resection was also collected. After nucleic acids extraction, NGS analysis

was performed on each timing point by using a custom NGS panel able to target most recurrent clinically relevant alterations in mCRC predictive biomarkers in order to track minimal residual disease (MRD) by inspecting variation in the mutated allele frequency (MAF). [54] Moreover, patients that could not exhibit molecular alterations in predictive biomarker covered by custom NGS assay will be then analyzed with a commercially available panel (Oncomine Colon *cell-free DNA* Assay, Thermo Fisher Scientific,USA) able to perform an extensive molecular analysis in liquid biopsy specimens of mCRC patients. Molecular results obtained from liquid biopsy specimen were then compared with the corresponding molecular results performed on the matching formalin fixed paraffin embedded samples (FFPE) in order to evaluate mutation allele frequencies (MAF) variations in the four blood specimens and to monitor relapse after surgical resection by using a molecular highly sensitive approach. Patient's data and biologic materials were managed according to Helsinki declaration.

Tissue analysis

DNA extraction and purification

In this preliminary report, a total of n=15 FFPE samples of mCRC patients with proven liver metastases were retrospectively collected from our laboratory (Predictive Molecular Pathology Laboratory, Department of Public Health, University Federico II of Naples). As regards, genomic DNA (gDNA) was extracted using the QIAamp DNA Mini Kit (Qiagen, Crawley, West Sussex, United Kingdom) according to the manufacturer's instructions. Briefly, after DNA extraction and purification with proprietary salt buffer, DNA was finally resuspended in 30 µl of *Not-DEHPC* water. Purified nucleic acids were immediately evaluated or stored at -20C^{o} until nucleic acids quantification and analysis.

Plasma analysis

cf DNA isolation

For each timing point cfDNA was purified as follows: a total of n=10 ml of peripheral blood was withdrawn from each patient and collected in n=2 Vacutainer tubes (BD, Plymouth, UK). Plasma was immediately centrifuged twice at 2300 rpm for 10 min in order to separate supernatant (that contains cfDNA) from cell debris. The plasma supernatant was then aliquoted and used immediately for cfDNA isolation or stored at -80 °C until sample processing. On the overall, 1.2 ml of purified plasma was adopted to isolate cfDNA for each experimental point. In the rare instances that the volume of the plasma ranged between 1 and 1.2 ml, PBS up to 1.2 ml was added to the samples. An automatized platform (QIA symphony, Qiagen) was then used to extract and purify cfDNA following manufacturer instructions. Extraction workflow was optimized by using QIAsymphony DSPVirus/Pathogen Midi Kit as previously demonstrated. [59] Finally, cfDNA was eluted in a final volume of 60µl and immediately evaluated or stored at -20Cº until nucleic acids quantification and analysis.

Nucleic acids analysis (qualification and quantification)

A microfluidic platform (TapeStation 4200) was adopted to qualify and quantify extracted nucleic acids (both gDNA and cfDNA). In details, 1µl of extracted DNA was automatically processed on the instrument. Genomic Reagents (Agilent) and Genomic ScreenTape (Agilent) were combined to successfully carry out molecular results. For each sample, nucleic acids quantification (ng/µl) and fragment index evaluation inspected as DIN (DNA Integrity Number) was evaluated with proprietary software.

NGS analysis

Libraries were constructed and purified on the automatized robot (Ion Chef, Thermofisher). In details, library generation was performed as follows: 15 μl of DNA (for a total of 15 ng of cfDNA or gDNA) were dispensed on Ion Code plates and amplified using Ion AmpliSeq DL8 (Thermofisher). We used 26 cycles for DNA amplification and 4 cycles for library reamplification after barcoding, under the thermal conditions defined by the manufacturer. Purified libraries were diluted and combined with the remaining DNA-derived libraries to obtain pooled library. The two-pooled libraries were re-loaded into the Ion Chef instrument, and templates were prepared using the Ion S5 Kit (Thermofisher). Finally, templates were loaded into the 520™ chip and sequenced on the IonTorrent™ & GeneStudio™ S5 Plus System (ThermoFisher).

2.3 Results

Overall, molecular analysis was successfully performed in matched tissue specimen for a preliminary set (n=15) of enrolled patients. In n=3 cases, gDNA was not available for molecular analysis. In details, tissue sample analysis showed at least a molecular alteration in n=8/12 (66,6%) cases. Among them, n=4/12 (33,3%) patients harbored a clinically relevant alterations in key genes for the pathogenetic mechanism of mCRC while a polymorphic alteration was detected in other cases. (Table 3) As regards, *KRAS* exon 2 point mutation (p.G12V; 75%) and *NRAS* exon 3 point mutation (p.Q61R;10,0%) was detected in n=3 (25,0%) and n=1 (8,3%) cases, respectively. A single case (#2) highlighted a concomitant clinically relevant alteration in *KRAS* gene (p.G12V; 41,7%) and a polymorphic variant in *cKIT* (p.M541;48,5%) and *PDGFRα* (*PDGFRα* p.V824V;53,3%) genes. In the remaining cases a single molecular alteration was identified.

T0 molecular analysis

Molecular analysis was carried out in all instances. Among them, n=2/15 (13,3%) patients harbored a clinically relevant alterations in key genes for the pathogenetic mechanism of mCRC while a polymorphic alteration was detected in n=6 cases [n=2/6 33,3% and n=3/6 50,0% harbored *c KIT* p.M541 and *PDGFRα* p.V824V and n=1/6 16,6% harbored both mutations] with a similar MAF respect to corresponding tissue specimens. (Table 3) As regards, a *KRAS* exon 2 point mutations in (p.G12V;50%) was detected in n=2 cases. A single case (#2) highlighted a concomitant clinically relevant alteration in *KRAS* gene (p.G12V;7,5%) and a polymorphic variant in *cKIT* (p.M541L;43,2%) and *PDGFRα* (p.V824V;53,3%) genes. In the remaining cases a single molecular alteration was identified. In addition, an exon 11 p.G469E *BRAF* point mutation (#3) and exon 20 p.H1047L *PIK3CA* point mutation was also detected (#6). (Table 3)

T1 molecular analysis

Molecular analysis was carried out in all instances. Among them, n=1/15 (6,6 %) patients harbored a clinically relevant alterations in key genes for the pathogenetic mechanism of mCRC while a polymorphic alteration was detected in other cases [2/6 33,3% and n=3/6 50,0% harbored *cKIT* p.M541; *PDGFRα* p.V824V and n=1/6 16,6% harbored both mutations] with a similar MAF respect to corresponding tissue specimens (Table 3) As regards, a *KRAS* exon 2 point mutations in (p.G12V;9,4%) was detected in n=1 case.

T2 Molecular analysis

Molecular analysis was carried out in all instances. In each case *wild type* molecular assessment was also detected. In addition, a polymorphic alteration was respectively detected in all patients that

showed the corresponding molecular alteration at basal setting. In details, n= 4/15 (26,6%) showed *PDGFRα* polymorphic alteration (p.V824V) as reported in the Table 3 and n=2/15 (13,3%) showed the *cKIT* polymorphic alteration (p.M541L) as reported in the Table 3.

ID patients	FFPE tissue samples	Liquid Biopsy T0 (pre-surgical)	Liquid Biopsy T1 (post-surgical)	Liquid Biopsy T2 (30 days post-surgical)
1	WT	WT	WT	WT
2	KRAS p.G12V (41,7%) KIT p.M541 (48,5%) PDGFRα p.V824V (53,3%)	KRAS p.G12V (7,5%) KIT p.M541L (43,2%) PDGFRα p.V824V (53,5%)	KRAS p.G12V (9,4%) KIT p.M541L (34,1%) PDGFRα p.V824V (42,9%)	PDGFRα p.V824V (49,1%)
3	NA	BRAF p.G469E (3,9%) KIT p.M541L (48,8%)	KIT p.M541L (53,2%)	KIT p.M541L (59,2%)
4	KIT p.M541L(48,3%)	KIT p.M541L(53%)	KIT p.M541L (49,9%)	KIT p.M541L (52,5%)
5	NA	PDGFRα p.V824V(47,1%)	PDGFRα p.V824V (52%)	PDGFRα p.V824V (51,7%)
6	PDGFRα p.V824V (53,3%)	PI3KCA p.H1047L(33,5%)	WT	WT
7	NA	WT	WT	WT
8	NRAS p.Q61R(10%)	WT	WT	WT
9	WT	WT	WT	WT
10	WT	WT	WT	WT
11	PDGFRα p.V824V (49,3%)	PDGFRα p.V824V (45,4%)	PDGFRα p.V824V (48,1%)	PDGFRα p.V824V (48,4%)
12	PDGFRα p.V824V (47,6%)	PDGFRα p.V824V (45,4%)	PDGFRα p.V824V (48,4%)	PDGFRα p.V824V (49,3%)
13	KRAS p.G12V (51,2%)	KRAS p.G12V (2,1%)	WT	WT
14	KRAS p.G12V (40%)	WT	WT	WT
15	WT	WT	WT	WT

Table 3. Results of 15 mCRC patients tested with NGS approach: 15 liquid biopsy pre-surgical samples (T0), 15 liquid biopsy post-surgical samples (T1), 15 liquid biopsy after 30 days of surgical resection (T2) and theirs matching 15 FFPE gold standard tissue samples.

2.4 Discussion

In this retrospective study, we investigated a novel liquid biopsy-based workflow for the evaluation of MRD in a perspective cohort of mCRC patients. Conventional technical approaches routinely adopted in the decision making of mCRC patients with liver metastasis after surgical treatment are characterized by a very low sensitivity in the identification of MRD.[56] In fact, a not negligible number of patients relapse in few years starting from surgery as therapeutic choice. In this scenario, the identification and validation of a novel approach based on the molecular features evaluation of pathogenetic mechanisms at the basis of mCRC is necessary to optimize clinical administration of diagnostic patients. [56] As regards, liquid biopsy is considered an emerging and pleiotropic tool yet approved in the selection of NSCLC patients for TKIs administration that may cover a crucial role in the management of mCRC patients. [57] This scant source of nucleic acids requires high sensitivity technology enables to identify very low frequency alterations harbored by this kind of sample. [58]
An integrated workflow based on the adoption of Real Time PCR and NGS systems was used by *Lupakis et al.* in order to improve MAF detection rate in early stage and follow up of CRC patients. In order to improve the detection rate of molecular alterations mainly involved in the molecular mechanisms of CRC, our workflow was based on an integrative approach where a two target NGS panels were implemented [56]. *Yaegashi et al* evaluated a multiregional targeted NGS approach in order to overcome spatial limitations. [58]. In this project, a custom NGS panel able to cover clinically relevant hot spot in *KRAS, NRAS, BRAF,* and *PIK3CA* genes was used as a screening tool to exhaustively analyze most frequent alterations (50-60%) found in CRC patients. This approach allows

to reduce technical costs derived from NGS platforms. In addition, a wide NGS commercially available panel will be adopted to improve detection rate of less frequent pathogenetic alterations identified in non-recurrent mutated genes. This approach will determinate a comprehensive molecular analysis able to detect a large spectrum of molecular alterations in key genes for CRC pathogenesis. In this preliminary study, a clinically relevant alteration was found in T0 of n=4 patients (27%). Our NGS panel was capable to evaluate MRD in T2 and T3. Interestingly, Patients #2 and #13 harbored exon 2 p.G12V *KRAS* mutation. In all instances, MAF decreasing was observed in T0 while WT status was identified in T1 and T2 sample. Patients #8 and #14 showed exon 3 p.Q61R *NRAS* and exon 2 p.G12V *KRAS* alteration, respectively. Of note, only tissue specimen highlighted reported alterations while wild-type molecular assessment was identified in T0, T1 and T2 specimens. Finally, patient #1 simultaneously highlighted exon 2 p.G12V *KRAS* and n=2 polymorphic variants in *PDGFRα* and *cKIT* genes. In this case, a MAF variation was only detected among tissue, T0 and T1 samples for *KRAS* mutations while no significant variation was remarkably detected for polymorphic variants. In the remaining patients (n=4) where a unique polymorphic variant was inspected, frequency alteration was stable among different sampling modalities because therapeutic strategy does not impact on these germline alterations characterized by a low clinical impact on CRC patients. Of note, in #3 case exon 20 p.H1047L *PIK3CA* point mutation was only detected in T0 sample whereas no clinically relevant alterations were detected in corresponding tissue specimen. According to literature data, molecular heterogeneity in tissue specimens has a crucial impact on the molecular assessment evaluation. In this scenario, liquid biopsy specimen is the diagnostic source of nucleic acids able to overcome this tissue limitation. In conclusion, this preliminary data highlights that MRD may be considered a potential tool in the clinical management of relapsing mCRC patients and an integrating NGS workflow is essential to correctly administrate liquid biopsy specimens in this clinical setting.

Chapter 3

3.1 Fully Automated Real Time PCR approach (Idylla™) for *RAS* and *BRAF* mutation detection in clinical practice of mCRC patients: a prospective study.

The monoclonal antibodies that target and inhibit epidermal growth factor receptor (*EGFR*) are a major therapeutic option in metastatic colorectal cancer (mCRC).[59] Fully human monoclonal antibody such as Panitumumab (Amgen, Thousand Oaks, CA- USA) and Cetuximab (Merck KGaA, Darmstadt, Germany) , target the *EGFR* gene and can be used as monotherapy or combined with chemotherapy.[60] Earlier studies strongly suggested that the most common activating mutations in *KRAS*, in exon 2 codons 12 and 13, were negative predictors for treatment benefit from *EGFR* antibodies. Since 2009 *KRAS* gene testing is mandatory in mCRC patients in the USA, Europe and Japan, and the use of *EGFR* monoclonal antibodies is restricted to codon 12 and 13 wild-type tumors.[60] However, only a subset of *KRAS* codon 12 and 13 wild-type tumors benefit from *EGFR*-targeted therapy. This has led to intense investigation aimed at identifying additional markers of primary resistance. While no definitive predictive roles were identified for activating *BRAF* and *PIK3CA* mutations or for loss of *PTEN* expression, retrospective subset analyses of the data from phase II and III clinical trials broaded the definition of *RAS* mutations, [61-63] to include the so- called new, extended, or expanded *RAS* mutations: exon 3 codon 61 and exon 4 codon 117 and 146 in *KRAS* and exons 2, 3 and 4 in *NRAS*.

Expanding the analysis to all *RAS* mutations has, therefore, represented a paradigm shift in the testing strategy of molecular laboratories, with the increasing adoption of multiplex gene assays. Next generation sequencing (NGS) represents a viable option to carry out predictive colon cancer biomarker testing.[64] However, beside the *Turn Around Time* (TAT) of the molecular analysis process itself (about one week with NGS to date), this organization based on a centralized molecular test

increases the time between the prescription of a molecular analysis. The delivery of a clinically usable for treatment choices molecular result could delay the treatment of patients with advanced cancers, some of them suffering of acute deterioration and needing rapid therapeutic decisions.[60]

The Idylla™ automated technology (Biocartis, Mechelen, Belgium) has recently been reported in several studies 14,15 as a tool easy to be implemented in pathology laboratories to diagnose quickly and easily oncogenic mutations. Briefly, the Idylla™ system consists in a cartridge-based fully automated medical device able to perform molecular analysis in less than one day even in laboratory without a specific expertise in molecular analysis.

The study design

In this study n=450 cases of mCRC patients were prospectively collected to perform molecular analysis in target genes.

Afterwards, the molecular tests were carried out locally in a sizeable number (n.= 14) of Italian institutions, including centers located in the different part of the country, without needing molecular infrastructure or a specific expertise. In details, *KRAS* and *NRAS* mutational analysis are part of the routine diagnostic workup of patients with mCRC and these assays were performed by CE-IVD approved diagnostic test using the fully automatized Real-TimePCR Idylla™.

In detail, for each FFPE sample the Idylla™ *KRAS* Mutation Assay was performed. According to molecular status, *KRAS wild-type* patients were then analyzed with Idylla™ *NRAS-BRAF* Mutation Assay. Only *KRAS, NRAS, BRAF wild-type* samples will be then selected from Oncology Unit to be treated with anti-*EGFR* and treatment outcomes in terms of Progression-free survival (PFS) that will be defined as the time from the first administration of anti *EGFR*- monoclonal antibodies to the first evidence of disease progression or death from any cause; and in terms of Overall survival (OS) that will be considered the time from the first administration of anti *EGFR*- monoclonal antibodies to death from any cause, will be then reported.

A comprehensive written informed consent should be signed before to perform any testing procedure to assess to mutational status of *K-N-RAS* genes. All data regarding the human material were managed using anonymous numerical codes and all samples were handled in compliance with the Helsinki declaration. (Figure 3)

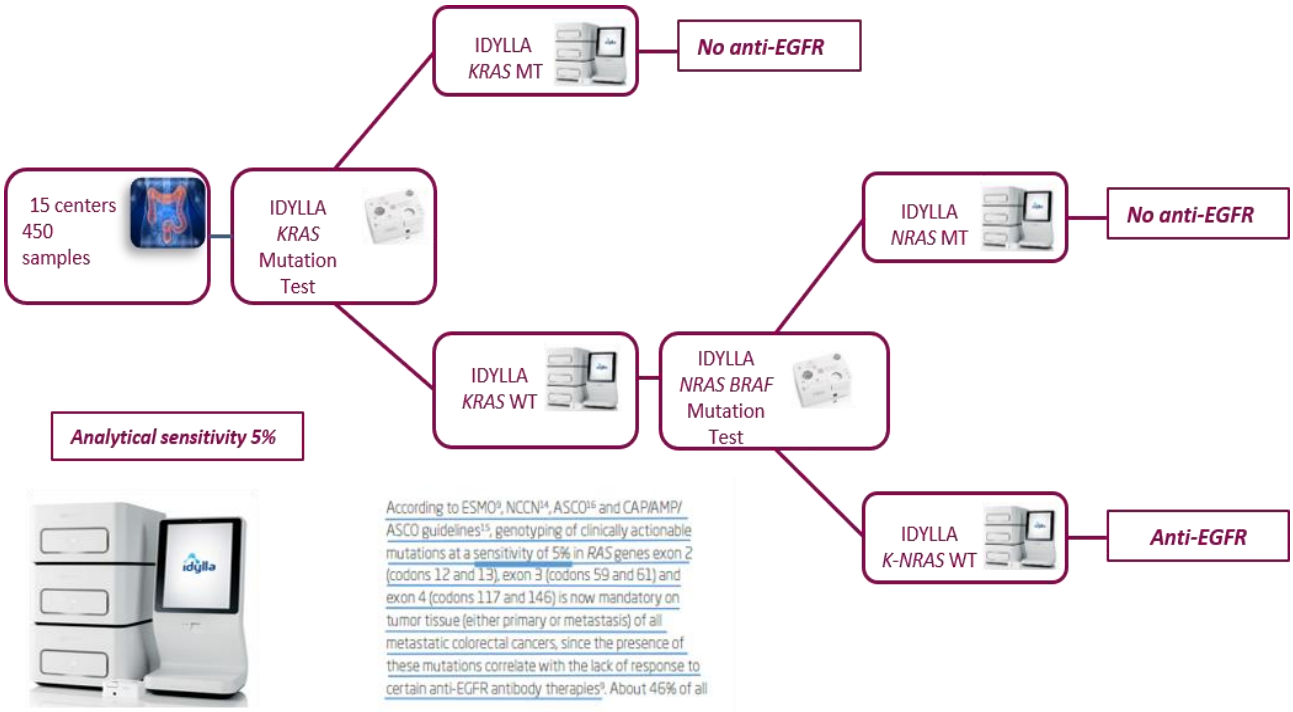

Fig. 3. Study design of our prospective study in real practice.

3.2 Material and methods

The Idylla™ System

The Idylla ™ system is a molecular diagnostic resource designed to detect genetic mutations in different types of carcinomas, such as in colorectal-cancer. This device is based on automated quantitative allele specific RT-PCR and it runs single tests at a time by entering the sample into a

cartridge loaded with all the reagents in dried form.[65] Furthermore, inserting the sample into a fully closed cartridge minimizes the risk of contamination. In addition, there is no need of experienced operators to use the instrument and to analyze data.

It is a fully-automated PCR system performing all necessary steps automatically. This reduces errors, hands-on time, risk of cross contamination and allows the fast and reproducible assessment of EGFR mutations comparable to main other techniques.[66] It allows the automatic generation of a report with the mutation status in less than one working day (~2 h). Briefly, it is possible to log in with a personal user account, do the QR code scan on the Idylla™ cartridge before adding sample information and place the cartridge in the Idylla™ system.

The Idylla™ Assay

The Idylla™ assay is validated for the use with the formalin-fixed and paraffin embedded tissue sections (FFPE) samples, that are placed in a single-use cartridge, itself placed in the Idylla™ module and every step of a Real-Time PCR are performed automatically inside the cartridge, from DNA extraction to an automated interpretation of the mutational status. Biocartis already trade two different assays focused on the detection of all hotspot mutations clinically relevant in *KRAS* and *NRAS* genes. These assays are approved in Europe for in vitro diagnostic use (CE-IVD).

Briefly, for the sample preparation there is the need to prepare two waterman filters and wet them shortly in laboratory grade water, then it can place them both on a microscope slide. It takes the stack of the FFPE slide between two waterman filters and add it directly into the specific Idylla™ cartridge and close the cartridge before to place the device in the Idylla™ system, follow the on-screen steps described by the system.

Patient's selection

Thanks to the collaboration between the Oncology and the Anatomo-Pathology Units, the 15 Italian Center involved into this study collected the tissue samples from mCRC patients enrolled. In details, the patients during the Oncology daily ambulatory, signed the informal consent for the study participation and it that have been forwarded to the Anatomo-Pathology Unit who evaluates the samples adequacy respecting the following criteria: (1) the sample should have an histological diagnosis of colorectal adenocarcinoma; (2) the availability of a tissue section, whose minimal size should be within 5 to 10 μm range 5 and with an adequate percentage of neoplastic cells >10%.

K- and *N-RAS* analysis

For this study a similar analytical workflow has been adopted in each involved laboratory; the *KRAS* and *NRAS* Idylla™ assays have been performed sequentially.

From an analytical point of view, the Idylla™ assay enable the accurate and sensitive detection of as low as 5% of mutant alleles.

In particular, the Idylla™ *KRAS* Mutation Assay enables mutation detection in exons 2, 3, and 4 of the *KRAS* oncogene. The assay consists of allele-specific multiplex PCR reactions, designed for the specific amplification of *KRAS* gene sequences with a mutation in codons 12, 13, 59, 61, 117, or 146. As such, the test enables detection of 21 *KRAS* mutations, i.e., seven mutations in exon 2 (codons 12 and 13), nine mutations in exon 3 (codons 59 and 61), and five mutations in exon 4 (codons 117 and 146) of the *KRAS* oncogene.

Only cases tested wild-type by the *KRAS* assay, have been undergoing to the Idylla™ *NRAS-BRAF* mutation test to detect 18 mutations in codons 12, 13, 59, 61, 117, 146 of the *NRAS* oncogene and also to detect, simultaneously, 5 mutations in codon 600 of *BRAF* gene. Figure 4

Fig. 4. Idylla™assay work-flow for *KRAS* and *NRAS-BRAF* testing.

3.3 Results

We enrolled 450 mCRC patients during three years of study. A total of 457 samples were tested in this prospective study in real practice; in particular, for 5 patients were analyzed also the metastatic tissue and the Idylla™ results perfectly match with the analysis on primary tumor tissue and for 2 patients we analyzed two synchronous cancers. In these latter patients, Idylla™ assay detected a *BRAF* mutation only in a single cancer of one out of two patients.

About the 14 involved Italian centers only 11 /14 participated actively to the patient enrollment.

In detail, the center #6 enrolled the largest number of mCRC patients n=114 and the center #11 the smallest number n=3 (*Table 4*).

A total of n= 200/457 (43,8%) samples gave a WT result, otherwise n=257/457 (56,2%) samples showed a mutation in the clinical relevant genes in patients with colorectal cancer.

In particular n=213/257 (82,9%) were *KRAS* positive, n= 20/257 (7,8%) were *NRAS* positive and n=24/257 (9,3%) were positive in *BRAF* gene.

The patient cohort was composed as follow: from the 450 enrolled mCRC patients, 278 cases (61,8%) were male and 172 cases (38,2%) were female. Their mean age was 67.94 (range: 34 to 95 years). The most common subsite of the primary tumor was the colon (n=234/450; 52,0%), and the overall of the tumors were adenocarcinoma (n=318/450; 70,0%) with grading G1-G3.

For all tested samples we reported a range from10 to 90 % of neoplastic cells content (average 60%).

ID Center involved	Region	Nr.patients	Samples tested	WT cases	KRAS MT	NRAS MT	BRAF MT
1	Campania_1	71	77	28 (36,4%)	40 (52,0%)	2 (2,6%)	7 (9,0%)
2	Abruzzo	47	47	19 (40,4%)	24 (51,0%)	2 (4,3%)	2 (4,3%)
3	Campania_2	39	40	14 (35,0%)	22 (55%)	1 (2,5%)	3 (7,5%)
4	Puglia_1	35	35	16 (45,7%)	13 (37,1%)	4 (11,4%)	2 (5,7%)
5	Piemonte	30	30	10 (33,3%)	15 (50,0%)	3 (10,0%)	2 (6,7%)
6	Puglia_2	114	114	54 (47,4%)	55 (48,2%)	3 (2,6%)	2 (1,8%)
7	Lazio	34	34	12 (35,3%)	21 (61,7%)	0 (0,0 %)	1 (2,9%)
8	Puglia_3	37	37	23 (62,1%)	11 (29,7%)	2 (5,4%)	1 (2,7%)
9	Lazio	34	34	18 (53%)	11 (32,3%)	3 (8,9%)	2 (5,9%)
10	Sicilia	6	6	6 (100%)	0 (0%)	0 (0%)	0 (0%)
11	Lombardia	3	3	0 (0%)	1 (33,3%)	0 (0%)	2 (66,7%)
Tot		450	457	200 (43,8%)	213 (46,6%)	20 (4,4%)	24 (5,2%)

Table 4. mCRC patients enrolled for IDEAL Study from each Italian Center and data obtained by performing the *KRAS* and *NRAS-BRAF* Idylla™ Mutation Assay.

3.4 Discussion

Nowadays one of the suitable therapeutic strategies that can be adopted in patients with stage IV metastatic colorectal cancer is represented by the administration of monoclonal antibodies (Cetuximab and Panitumumab) directed against the epidermal growth factor receptor (*EGFR*) when patients do not show clinically relevant alterations in *KRAS, NRAS and BRAF* genes. For this reason, molecular analysis of these biomarkers is crucial in order to select patients for treatment with Cetuximab and Panitumumab. Therefore, the oncologists need to know the *K-N-RAS* mutational status to address the patient to a tailored therapy. On the overall the *RAS* mutation frequency in colorectal cancer is remarkable. In detail, mutations in *KRAS* have been found in approximately 45% of mCRC patients and between 15 and 37% in early stages of mCRC patients. In addition, the concomitance mutations in *KRAS* and *BRAF* reduces progression-free survival (PFS) and overall survival (OS) in those patients, when compared with the clinical parametric of patients without mutations in the aforementioned genes. Nevertheless, it is important to highlight that not all patients with wild-type *RAS* give the same response rate to anti-*EGFR* treatments because other molecular mechanisms might be involved. Regarding the *BRAF* gene, it was reported that around 10% of mCRC patients can show a mutation. The most frequent mutation in *BRAF* (about 90% of cases), occurs in codon 600 of exon 15 of the *BRAF* gene (p.V600E) and, in most instances, is mutually exclusive with mutations in *RAS* gene. Moreover, some studies have shown that patients with *BRAF* mutations have a reduced survival limited to stages III and IV, attributing a negative prognostic role to the *BRAF* gene.

In this setting, we designed a prospective study for the treatment selection of patients with colorectal cancer. The IDEAL Study includes 14 centers, including our Predictive Molecular Pathology Laboratory (Department of Public Health, University Federico II of Naples) that is the center coordinator, in order to enroll n=450 mCRC patients. The aim of this study is to demonstrate the

feasibility of the fully automated multiplex Real Time PCR, Idylla™, for the assessment of mutational status of clinically relevant genes in metastatic colorectal cancer (*K-N-RAS*) with the advantage of performing a rapid on-site molecular evaluation.

This study requires a careful synergy between three different members of multidisciplinary team: pathologists, molecular biologists and oncologists, who work together to provide a clinical treatment *ad hoc* for the oncological patients.

Moreover, performing the *K-NRAS* Idylla™ analysis it will be possible in a short time-frame to address *wild- type* patients to the anti-*EGFR* treatment.

From enrolled 450 mCRC patients, n=457 samples were tested, because for n= 5 patients we collected both the primitive and the metastasis tissue and for n=2 patients we analyzed two synchronous cancers.

A total number of 457 samples tested with the Idylla™ Assay gave a valid result. In particular, n= 257 samples showed a mutation in the *KRAS, NRAS* and *BRAF* gene; conversely, we observed n=200/457 (43,8%) samples *wild-type*. In details, n= 213/457 (46,6%) samples were *KRAS* mutated, n=20/457 (4,4%) samples *NRAS* mutated and n=24/457 (5,2%) samples *BRAF* mutated.

The *wild type* samples tested with Idylla™ System belong to n=197 patients who have been addressed to the anti-*EGFR* treatment and for each one the oncologist will collect the treatment data in terms of PSF and OS.

The anti-*EGFR* treated patient cohort and the treatment collected data could be useful to evaluate the different response rate among *wild type* patients and also to verify, for those, the presence of mutations at a lower allelic frequency that cannot be detected by Idylla ™ System, using a more sensitive method such as the NGS.

All this combined data could be represented a milestone to address selected patients to the best personalized therapy.

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