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Article

The Role of Multigene Panel Next-Generation Sequencing Techniques in Managing Patients with Metastatic Colorectal Carcinoma

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Abstract: The efficacy and cost-effectivness of Multigene Panel Next-Generation Sequencing (NGS) in directing patients towards genomically matched therapies remains uncertain. This study investigated metastatic colorectal cancer (mCRC) patients who underwent NGS analysis on formalin-fixed paraffin-embedded tumor samples. Data from 179 patients were analyzed, revealing no mutations in 39 patients (21.8%), one mutation in 83 patients (46.4%), and two or more mutations in 57 patients (31.8%). *KRAS* mutations were found in 87 patients (48.6%), including *KRAS* G12C mutations in 5 patients (2.8%), *PIK3CA* mutations in 40 patients (22.4%), *BRAF* mutations in 26 patients (14.5%). Less common mutations were identified: *ERBB2* in 5 patients (2.8%) and *SMO* in 4 patients (2.2%). Additionally, *MAP2K1*, *CTNNB1*, and *MYC* were mutated in 3 patients (2.4%). Two mutations (1.1%) were observed in *ERBB3*, *RAF1*, *MTOR*, *JAK1*, and *FGFR2*. No significant survival differences were observed based on mutation numbers. 40% of patients had druggable molecular alterations, but only 1.1% received genomically guided treatment, suggesting limited application in standard practice. Despite this, expanded gene panel testing can identify actionable mutations, aiding personalized treatment strategies in metastatic CRC, although current eligibility for biomarker-guided trials remains limited.

Keywords: metastatic colorectal cancer; multigene panel; next-generation sequencing; molecular biomarkers; druggable targets

1. Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related death and accounts for 10% of cancer diagnoses worldwide [1]. Over the last decades, personalized decision-making has been empowered by the increasing use of comprehensive genomic profiling which has allowed the detection of novel actionable targets. As known, EGFR inhibition with specific monoclonal antibodies has become the standard of care in *RAS* wild type patients, providing significant benefits in terms of survival when administered in combination with chemotherapy[2,3]. More recently, specific *RAS* mutations such as *KRAS* G12C have risen interest as possible actionable targets, following the evaluation of sotorasib or adagrasib in combination with anti-EGFR.[4,5]. The actionability of other mutations such as *BRAF* V600E has also provided encouraging results, leading to the approval of the BRAF inhibitor encorafenib in combination with anti-EGFR cetuximab[6]. Larger aberrations such as *HER-2* amplification have also been investigated in several studies, evaluating the role of different combination strategies and providing interesting results[7–9]. Genomic profiling has thus widened the application of targeted therapy in digestive oncology[10,11] and also empowered the use of immune checkpoint inhibitors (ICI) whose efficacy is restricted to patients harboring microsatellite instability (MSI-H) and *POLE-D1* mutations[12,13]. Despite the encouraging results obtained in terms

of survival and disease control, targeted agents employment is burdened by primary and secondary resistance [11,12]. For this reason, their use remains restricted to a limited proportion of patients.

The advent of high throughput techniques such as DNA and RNA sequencing has allowed the global and unbiased evaluation of the genomic landscape of tumors, enabling the identification of hotspot mutations, copy number variants and fusion transcripts.

In this study, we analyzed a real-world cohort of mCRC patients, treated in our institution. All patients underwent NGS-multigene profiling for diagnostic and therapeutic purposes. Genomic and clinical data were collected in order to provide novel insights about epidemiological prognostic features.

2. Results

2.1. Patient Characteristics

Patients with a histologically confirmed diagnosis of colorectal adenocarcinoma and an NGS assay performed in our institution from June 2019 until December 2020, were included. A total of 179 patients were considered for the analysis.

Patient characteristics are summarized in Table 1. Male gender was the most represented (n= 105, 59%) and the median age at diagnosis was 68 years (IQ-IIIQ: 58.3-77.4, min-max: 30-89 years). Eastern Cooperative Oncology Group Performance status (ECOG PS) at metastatic disease diagnosis was recorded in 160 patients (89.3%), with 140 patients (78.2%) showing ECOG PS 0 or 1, and 20 patients (11.1%) with ECOG PS ≥ 2. Synchronous metastases were observed in 63.1% of cases (n=113), whereas metachronous lesions were identified in 36.9% of cases (n=66). A single metastatic site was identified in 115 patients (64.3%), whereas two or more metastatic sites were observed in 64 patients (35.7%). Surgical resection of metastases was performed in 28% of cases, with hepatic resections being the most common (30%), followed by peritoneal (20%) and pulmonary metastasectomies (18%). In total, 149 patients (83.2%) underwent first-line chemotherapy, with 51.9% of patients (n=93) receiving at least two lines of treatment afterwards. 44.1% of patients (n = 66) received doublet or triplet chemotherapy combined with bevacizumab, and 24.9% (37 patients) received doublet or triplet chemotherapy with an EGFR inhibitor (cetuximab or panitumumab). Chemotherapy alone was administered to 20.8% (31 patients) of cases, and 10% (15 patients) received the combination of 5-FU and bevacizumab or EGFR inhibitor. Eight patients with NGS-detected actionable alterations were administered targeted therapies. Among these patients, six harbored *BRAF* V600E mutation and received a combination of encorafenib and cetuximab, while two patients with *PI3K* alterations were treated with alpelisib, a PI3K inhibitor.

Table 1. Patient Characteristics.

Variable	n	(%)
Gender		
Female	74	(41.3)
Male	105	(58.7)
Median age at diagnosis (IQ-IIIQ)	69.6 (58.3-77.4)	
ECOG PS		
0	109	(60.9)
1	31	(17.3)
2 – 3	20	(11.2)
NA	19	(10.6)
Primary Tumor Site		
Right Colon	64	(35.7)
Left Colon	69	(38.6)
Rectum	46	(25.7)
Stage at diagnosis		
I-III	113	(63.1)
IV	66	(36.99
Number of Metastatic Sites		
1	115	(64.2)
>1	64	(35.8)

Metastatic Sites		
Liver	127	(70.9)
Lung	50	(27.9)
Lymph Nodes	34	(18.9)
Peritoneum	44	(24.5)
Bone	6	(3.3)
Pelvic Recurrence	6	(3.3)
Other	16	(8.9)
Metastasis Surgery		
Yes	50	(27.9)
No	129	(72.1)
Type of Metastasis Surgery		
Hepatic	15	(30.0)
Pulmonary	9	(18.0)
Peritoneum	10	(20.0)
Other Site	16	(32.0)
First-Line Systemic Chemotherapy		
Yes	149	(83.2)
No	30	(16.8)
First-Line Chemotherapy Regimen		
Doublet/Triplet plus bevacizumab	66	(44.1)
Doublet/Triplet plus EGFR inhibitor	37	(24.9)
Chemotherapy alone (Doublet or monotherapy)	31	(20.8)
Monotherapy + bevacizumab or EGFR inhibitor	15	(10.1)
Number of Treatment Lines		
1	54	(36.2)
2	40	(26.9)
3	33	(22.1)
4	16	(10.8)
≥ 5	6	(4.0)
First-Line Targeted Therapy		
Reimbursed	6	(3.3)
Clinical Trial	2	(1.1)

IQ: first quartile; IIIQ: third quartile; NA: not available; EGFR inhibitor: Epidermal growth factor receptor inhibitor (cetuximab or panitumumab). Percentages may not equal 100 due to rounding.

2.2. Molecular Alterations

No mutations were detected in 39 patients (21.8%) whereas 140 patients reported at least one mutation (78.2%), Figure 1. Table 2 shows the distribution of molecular alterations observed in the study population. The most frequent mutations were those in the *KRAS, PIK3CA,* and *BRAF* V600E genes. Some mutations were observed   only in 3 patients such as those in the *CTNNB1*, *MYC*, and *MAP2K1* genes. Other mutations where even more rare such as those in *ERBB3*, *RAF1*, *MTOR*, *JAK1*, and *FGFR2* genes detected each in only two patients or those in the in *CDK4*, *MET*, *FGFR3*, *GNA11*, *EGFR*, *ALK*, *ROS1*, *DDR2*, and *KIT* genes found in only one patient each. It is worth noting that no amplifications or fusions were identified and hotspot mutations were the only type of detected aberration (Single-Nucleotide Variants SNVs, and insertions-deletions INDELs). No mutations were observed for 28 genes of the Oncomine Focus panel, not shown.
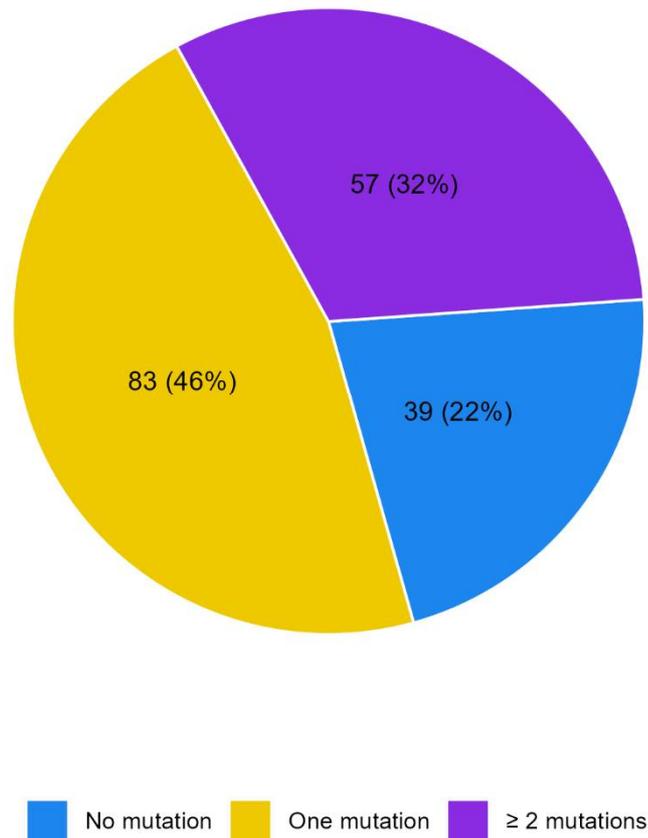
Figure 1. – Pie chart of the number of alterations per patient.

Table 2. Distribution of the type of molecular alterations.

KRAS	**87 (48,6%)**
PIK3CA	40 (22,4%)
BRAF	26 (14,5%)
APC	15 (8,4%)
NRAS	8 (4.5%)
ERBB2	5 (2.8%)
SMO	4 (2.2%)
CTNNB1, MYC, MAP2K1	3 (1,7%)
ERBB3, RAF1, MTOR, JAK1, FGFR2	2 (1.1%)
CDK4, MET, FGFR3, GNA11, EGFR, ALK, ROS1, DDR2, KIT	1 (0.6%)

The total does not add up to the total number of patients as the alterations are not mutually exclusive. As a secondary analysis, we observed the prevalence of actionable mutations, which was as follows: *BRAF* V600E 14.5% (n=26), *PIK3CA* 22.4% (n=40), *KRAS* G12C 2.8% (n=5), and *MET* 0.6% (n=1).

2.3. Association between Mutated Genes and Survival (PFS, OS)

Overall, the median follow-up time was 33 months (95% CI 28.45 – not reached - NR), with a median PFS of 10.3 months (95% CI 8.8 - 12.3) and a median OS of 32.7 months (95% CI 24.8 - 39.2). The study investigated the association between the   most frequent mutations, that is those occurring in at least 5% of patients, and PFS during first-line chemotherapy. Patients with a *KRAS*   mutation exhibited a median PFS of 10.4 months (95% CI 8.84 – 12.25), compared to 10.1 months (95% CI 7.29 – 13.70) in *KRAS* wild type patients (p=0.737; HR 0.95, 95% CI 0.69 – 1.29). Patients harboring *PIK3CA* mutation showed a median PFS of 10.7 months (95% CI 7.75 – 13.70), versus 10.0 months (95% CI 8.57 – 12.32) in *PIK3CA* wild type cases (p=0.942; HR 1.01, 95% CI 0.70 – 1.47). *BRAF* mutated tumors showed a median PFS of 5.2 months (95% CI 3.52 – 9.86) compared to 10.7 months (95% CI

9.00 – 12.94) in *BRAF* wild type (p=0.060; HR 1.54, 95% CI 0.98 – 2.40) (Figure 2, left panel), and *APC* mutated tumors demonstrated a median PFS of 9.0 months (95% CI 1.81 – 12.32) versus 10.3 months (95% CI 8.84 – 12.52) in *APC* wild type (p=0.330; HR 1.33, 95% CI 0.75 – 2.35).

The study also investigated the potential association between OS and the most frequently mutated genes. Analogous analyses for OS showed that patients with mutated *KRAS* exhibited a median survival of 34.9 months (95% CI 24.77 – 65.60) compared to 26.9 months (95% CI 22.37 – 39.19) in wild-type *KRAS* (p=0.084; HR 0.71, 95% CI 0.48 – 1.05). Similarly, mutated *PIK3CA* displayed a median OS of 35.8 months (95% CI 23.85 - NR) versus 30.2 months (95% CI 24.01 – 39.19) in wild-type *PIK3CA* (p=0.270; HR 0.76, 95% CI 0.47 – 1.24). In contrast, mutated *BRAF* showed a median OS of 13.9 months (95% CI 4.89 – 26.05) versus 35.8 months (95% CI 26.91 – 48.59) in wild-type *BRAF* (p<0.001; HR 2.62, 95% CI 1.59 – 4.32) (Figure 2, right panel). Additionally, mutated *APC* demonstrated a median OS of 24.8 months (95% CI 2.3 - MR) versus 32.8 months (95% CI 24.8 – 41.8) in wild-type *APC* (p=0.354; HR 1.36, 95% CI 0.71 – 2.62).

Among the less mutated genes, no differences were observed except for *CTNNB1* gene, however mutated in only three patients. Patients harboring a mutation in such gene showed a statistically significant shorter PFS and OS compared to wild type patients. In detail, patients with mutated *CTNNB1*, had a median PFS of   1.8 months (95% CI 1.15 - NR) compared to 10.4 months (95% CI 9.0 – 12.32) in *CTNBB1* wild type patients (p=0.001; HR 6.85, 95% CI 2.13 – 22.03). Similarly, the median OS in patients with mutated *CTNNB1* was 2.1 months (95% CI 1.87 - NR) versus 32.8 months (95% CI 24.8 – 41.8) in *CTNBB1* wild type (p<0.001; HR 9.82, 95% CI 3.00 – 32.12), Figure S1.

No significant differences in terms of OS and PFS were found when comparing patients with high KRAS or BRAF allele frequency, defined as Variant Allele Frequency (VAF) more than median value, versus low allele frequency (Figure S2, Figure S3).

No significant relationship was found between the number of mutated genes and survival, results not shown.

2.4. Association between RAS Mutation Allele Frequency and Survival

We also investigated the association between *RAS* mutation (*KRAS* and *NRAS*) allele frequency and survival outcomes in patients treated with first-line chemotherapy (n= 148). Patients with a high *RAS* allele frequency, defined as Variant Allele Frequency (VAF) > 20%, showed a trend for lower survival rates with a median OS of 30.95 months (95% CI 18.2-47.4)   compared to 37.42 months (95% CI 26.97-NR) for patients with low VAF (p-=0.091; HR=2.13, 95% CI 0.89-5.09), as shown in Figure S4.
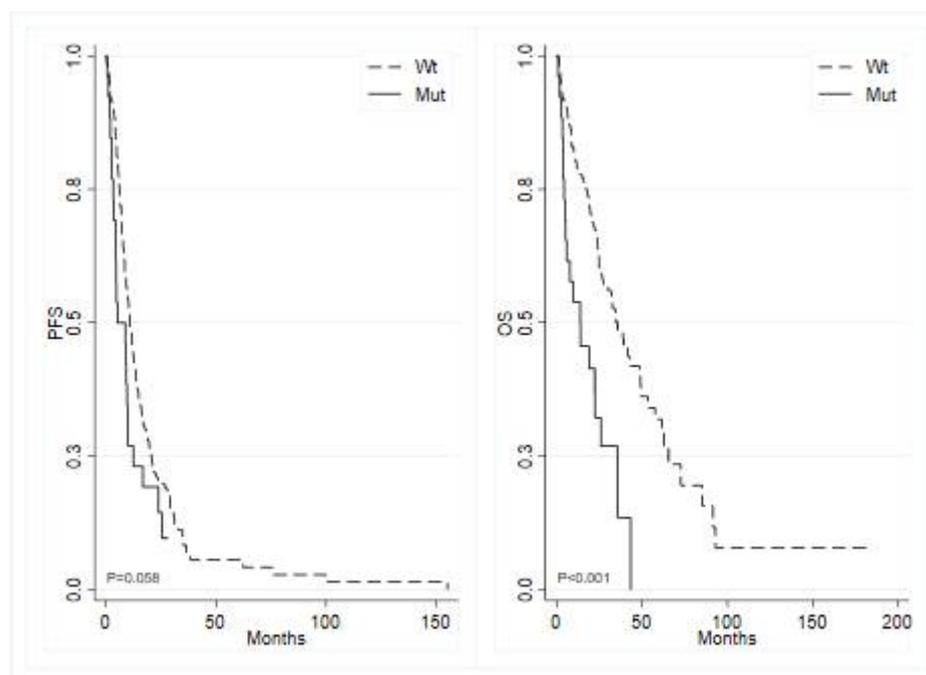

Figure 2. - Kaplan-Meier curves for progression-free survival (left) and overall survival (right) based on the mutational status of *BRAF* gene.

3. Discussion

To the best of our knowledge, there are currently few real world evidence studies based on routinely collected data from standard clinical practice that investigate the impact of NGS on a large cohort of patients diagnosed with mCRC. One of the main objectives of this study was to assess the clinical value of genomic profiling using NGS techniques in a sample of treated patients referred to our oncology units.

No new genomic prognostic factors were identified with this NGS panel, and *BRAF* V600E mutation is confirmed as a negative prognostic factor as previously reported in the literature [14]–[16]. While no statistically significant correlation was identified between the number of mutated genes and survival, there appears to be a negative survival trend in patients with at least one mutation. A statistically significant decrease in both PFS and OS was noted among patients harboring a mutation in the CTNNB1 gene. Nonetheless, due to the limited number of patients exhibiting this mutation, the reliability of the data, despite its statistical significance, has to be carefully interpreted, and no conclusive inferences can be drawn concerning its prognostic role.

The choice of the first-line therapeutic strategy was not influenced by NGS results. Indeed, in daily practice, the choice of the first line treatment is based only on the mutational status of *KRAS* and *BRAF* as well as microsatellite instability. Therefore, NGS data were taken in consideration only at progression following the discontinuation of standard treatments, in order to allow enrollment in a clinical trial or the use of off-label or expanded-access therapies. Unfortunately, many of these heavily pretreated patients were unfit for subsequent lines of treatment. This may explain the low number of patients treated with molecular-targeted therapies.

Molecular-targeted therapy in mCRC is increasingly becoming a therapeutic option due to the availability of selective inhibitors. Therefore, centralizing NGS genomic profiling in high-volume reference laboratories and establishing teams of professionals capable of evaluating and interpreting complex molecular test results (Molecular Tumor Board) is increasingly necessary to identify molecular alterations that provide sensitivity to specific molecular-targeted therapies in subpopulations of otherwise difficult-to-treat patients.

This study has some limitations that are worth noting. First, molecular analysis was performed on the primary tumor or metastasis without taking into account, as per clinical practice, the spatial and temporal heterogeneity of colorectal cancer. One limitation of this study is also related to the employed NGS panel which was restricted to 52 genes. Broader panels with the ability to analyze hundreds of genes can indeed identify a greater number of druggable molecular alterations, define the tumor mutational burden, and detect increasingly informative mutations such as *POLE/D1* mutations which are predictive of sensitivity to immunotherapies.

4. Materials and Methods

4.1. Patient Selection and Data Collection

Patients aged 18 years and older diagnosed with mCRC who were NGS-tested with the Oncomine Focus Assay between June 2019 and December 2020 at the Biosciences Laboratory of IRCCS Istituto Romagnolo per lo studio dei Tumori (IRST)were included. Patients with other concurrent neoplasms or localized colorectal carcinoma were excluded.

For each included patient, demographic and clinical data were collected. Additionally, molecular information such as the quantity and type of molecular alterations detected as well as the variations of their allelic frequency (VAF) were recorded.

All participants provided written informed consent, and the study was conducted in compliance with the Declaration of Helsinki under good clinical practice conditions and with approval from the Local Ethics Committee (Comitato Etico Area Vasta Romagna e IRST). Information was extracted from patients' electronic medical records in accordance with stringent privacy standards, and the data were anonymized and recorded in an Excel database.

4.2. Molecular Analyses

Formalin-fixed-paraffin-embedded (FFPE) tumor samples were used for molecular analysis. For each sample, tumoral areas were defined by the pathologists, macrodissected and collected in specific tubes.   Nucleic acids were extracted using MagMAX FFPE DNA/RNA Ultra Kit (Applied

Biosystems, Waltham, MA, USA) following the manufacturer's protocol. DNA and RNA concentrations were determined by fluorometric quantitation using a Qubit 4.0 Fluorometer with Qubit DNA dsDNA HS Assay Kit and Qubit RNA HS Assay Kit (Thermo Fisher), as appropriate.

NGS analyses were performed by Oncomine™ Focus Assay panel (Thermo Fisher Scientific Waltham, MA, USA), an amplicon-based DNA/RNA NGS assay that covers 52 cancer-associated genes. The DNA panel could identify hotspot mutations in 35 genes and copy number variants in 19 genes. The RNA panel was able to detect fusion drivers in 23 genes.

DNA and RNA libraries preparation were performed automatically using the library preparer "Ion ChefTM System" (Thermo Fisher Scientific) following the manufacturer's instructions, with 10 ng of input DNA and RNA per sample.

Prior to RNA library preparation, complementary DNA (cDNA) synthesis was carried out using SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific). The libraries were loaded onto Ion Chef System (Thermo Fisher Scientific) for template preparation and finally sequenced on the Ion S5 Plus platform (Thermo Fisher Scientific) using the Ion 520 Chips (Thermo Fisher Scientific). Primary analysis was carried out using a Torrent Suite Server™ to perform initial quality control, including chip loading density, median read length and number of mapped reads. Afterwards, a second analysis was performed by Ion Reporter™ Software, hosting informatics tools for variants, filtering, and annotations. Only variants with Variant Allele Frequency (VAF) greater than or equal to 5% and coverage greater than 500X have been reported. In addition, reliable variants are also reported if they have a relevant therapeutic significance, based on the sample pathologies in which they have been detected.

4.3. Statistical Analysis

A dedicated database was created to retrospectively collect demographic, clinical, histological, and treatment data. Patients characteristics were summarized using median and first- and third quartiles for continuous variables, and frequencies and percentages for categorical variables. PFS was defined as the time in months from the date of metastatic tumor diagnosis to progression or death from any cause, whichever occurred first, during the course of first-line chemotherapy. For patients alive without progression, the date of the last follow-up was recorded. OS was determined as the time in months from the date of metastatic tumor diagnosis to death from any cause or the date of the last follow-up. The median follow-up time was computed using the reverse Kaplan-Meier method whereas the survival curves were estimated using the Kaplan-Meier method, and comparisons were made using the log-rank test.. Univariate Cox regression models were employed to estimate hazard ratios (HR) and their corresponding 95% confidence intervals (CIs). With regard to the analysis of the association between the mutational status together with the variant allelic frequency (VAF) and survival, the cut-off were based on the median value of the VAF distribtuion of a specific gene excluding the wild type patients. Results were considered statistically significant if the two-sided p-values were <0.05. Statistical analyses were conducted using STATA 15.0 (College Station, TX, USA).

5. Conclusions

At present, molecular testing for MMR status as well as *KRAS*, *NRAS*, and *BRAF* mutations is recommended for all mCRC patients at diagnosis, according to ESMO Guidelines[17]. For what concerns CRC, all of the level 1 genomic alterations according to ESCAT (ESMO Scale for Clinical Actionability of molecular Targets), are represented by point mutations which can be easily identified by PCR. For this reason, ESMO recommendations for the use of NGS suggest genomic sequencing as a possible alternative to PCR only when it does not generate extra costs. Indeed, potentially actionable alterations are considered a rare event in CRC and the systematic use of NGS would not result cost-effective from a large-scale perspective. Nonetheless, unlike PCR, comprehensive genomic profiling increases multiplexing ability, analyzing multiple genes in a single test and providing information about different types of genomic aberrations. In this manner, NGS can provide vital information both for clinical and for research purposes by providing novel insights into patients' prognosis and resistance mechanisms. Even the enrollment in clinical trials could be hopefully increased following a wider accessibility to NGS platform, providing patients an easier access to novel, promising drugs. Comprehensive genomic profiling is feasible and cost-effective, when performed in reference centers.

Thus, a major effort is requested to local authorities in order to promote collaboration networks and facilitate information and tissue exchanges between peripheral and reference institutions.

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