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ABSTRACT

In metastatic stage, therapeutic approach for malignant melanoma is particularly based on performance status, metastatic sites, and *BRAF V600* status (*BRAF V600E/V600K* or *V600R* (class I *BRAF* mutations). In most cases, *BRAF* mutations and *NRAS* mutations are mutually exclusive to each other. However, some rare *BRAF* mutations class III are preferentially associated with a *NRAS* mutation, leading to the MAP Kinase pathway activation and subsequent cell proliferation. Melanomas with this double mutation are rare and difficult to treat because of the lack of codified therapeutic options. We report a patient with metastatic melanoma, harboring class III *BRAF* mutation (N581K) associated to *NRAS* mutation (Q61L) with treatment failure. He was treated in second line, after immunotherapy, by monotherapy of MEK inhibitor (MEKi), which underline the interest of NGS (Next Generation Sequencing) to early identify all mutations and enabling onco-dermatologist to discuss a treatment. Rare *BRAF non V600* mutations represent 3 to 14% of melanoma mutants and the aim of this communication is to promote the next generation sequencing to extend the paradigm of individually therapeutic approach with target therapy into different spectrum of melanoma patients.

1. Introduction

Historically, *NRAS* mutation was the first to be discovered, found in approximately 20% of the cases. It is usually associated with nodular histological subtypes and melanomas located on limbs. Most common mutations are *Q61R* and *Q61K* [1].

Among effectors of *NRAS*, MAP Kinase pathway is a well-established driver of melanoma [2], and *BRAF* mutations are present in approximately 50% of cases [3], preferentially in melanomas located on the torso and superficial spreading melanoma (SSM) histological subtypes [1]. *BRAF V600E* represents 85% of *BRAF V600* mutations [4]. Historically, *NRAS* and *BRAF* mutations were described as mutually exclusive [5], but the advances of genetic knowledge and the wider genetic screening are reeling us away from this old

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truth.

Metastatic melanoma therapy is based on performance status, staging, metastatic sites, and *BRAF* status. When tumor cells harbor class I *BRAF V600* mutation (*BRAF V600E/V600K* or *V600R*) patient can be treated with immunotherapy (PD-1 inhibitor with or without CTLA-4 inhibitor), or targeted therapy *BRAF* inhibitor + *MEK* inhibitor (BRAFi + MEKi) in first, or second line. However, when *BRAF V600* is wild type (WT) i. e non-mutated, only immunotherapy can be used, before conventional chemotherapy [6].

Currently, more than 200 alleles of *BRAF* mutations are known [7]. Different *BRAF* classes of mutations are described, and rare *BRAF non V600* mutations represent 3 to 14% of malignant melanomas [8].

BRAF class I mutations (*BRAF V600E/V600K* or *V600R*) correspond to *BRAF V600*, leading to a monomer with an activity 500 fold superior to wild type *BRAF* [5], causing enhanced activity of the MAP Kinase pathway through a *RAS* – independent signal. They are responsive to *BRAF* inhibitors (BRAFi): vemurafenib, dabrafenib, encorafenib.

BRAF class II mutations represent non-V600 mutations with dimers that are 138 times more active compared to wild type [8]. This class is divided into two subtypes: class IIa that are sensitive to BRAFi, and class IIb that are resistant to BRAFi [3]. The difference between both resides in conformational specificities [9].

These two first classes mutations (class I and II) are rarely associated with *NRAS* mutations.

Class III mutations are non-V600 mutations resulting in low activity or inactive kinase; they lead to the formation of CRAF-BRAF heterodimers, which activate the RAS dependent signal pathway through ERK retro-control. The mechanism remains poorly understood [7,10]. This class of mutation is also not responsive to classic BRAF inhibitors [3].

It is important to note that class III *BRAF* mutations are frequently associated with *NRAS* or *NF1* mutation in malignant melanoma [11].

There is no codified strategy to treat metastatic melanoma expressing *BRAF* non V600 mutation, associated to *NRAS* mutation. The aim of this communication is to promote NGS to propose targeted therapies and an individually therapeutic approach in melanoma and more broadly in oncology, making it possible to offer increasingly personalized care since 50–80% of non-small lung cancer, 20 to 30% of colorectal tumors, 9% of sarcoma, 40 to 60% of thyroid cancers, and according to series, 1 to 20% of biliary tract cancers harbored V600 or non V600 BRAF mutations, and so could be eligible to adapted targeted therapies [12–17].

2. Materials and methods

2.1. Next generation sequencing of circulating DNA

Circulating DNA was extracted from 2 to 4 ml of plasma using Maxwell® RSC cfDNA plasma large volume AS1840, on a Maxwell RSC 48 automated system (Promega™) according to the manufacturer's recommendation. DNA concentration was measured with Quantus Fluorometer (Promega).

NGS (next generation sequencing) was performed using GeneStudio S5 Prime (Thermo Fisher Scientific). The average depth was >1000X; on target >90%. Bioinformatic Analyses (Alignment, variant calling and an-notations) were run on LifeTechnologies: Torrent suite 5.10, Variant caller 5.10, Ion reporter 5.10. The CNV (Copy Number Variant) analysis was expressed as the ratio of mean depths by amplicons ± 2 standard deviations. The detection limit was set to 3% for SNV (Single Nucleotide Variant) and 5% for INDEL (insertion/deletion) for a minimum depth of 100X per amplicon. Variations of sequences recognized as non-pathogenic (class I and, class II variations according to ACMG classification, [18]) were not mentioned. The VAF (allelic frequency of variation) of an alteration is evaluated.

This method shows a great sensitivity with a 3% threshold for hotspot mutations and 5% for others mutations at diagnosis. Detection threshold could be lowered at 1–2%. Nevertheless, that is not a quantitative method and macro genetic events (as deletions, insertions/duplications and chromosomal rearrangements) are not detected.

3. Results

3.1. Case report

A 64-year-old man, Fitzpatrick II, with a history of chronic sun exposure, presented a stage IV melanoma in September 2016, without cutaneous or mucosal primitive lesion. Metastatic sites at diagnosis were lungs (bilateral multinodular lesions and one 15 mm nodule in the right inferior lobe), liver (multinodular), and node (right hilar, 16mm).

Diagnosis was confirmed histologically through punch biopsy of a liver lesion. Biopsy was not performed on other sites. Immunohistochemical (IHC) and molecular biology techniques did not find *BRAF* V600E/V600K or V600R mutations. Exploration was not pushed further and next generation sequencing (NGS) was not performed.

Patient was first treated by immunotherapy association: anti PD-1 (Nivolumab) combined with anti CTLA-4 (Ipilimumab) every 3 weeks (started in November 2016), for 4 cycles, resulting in partial response on pulmonary and liver targeted lesions (−66%) in February 2017. This sequence was followed by a monotherapy of Nivolumab 240 mg, every 2 weeks according to benchmarking practice (Checkmate 067) [19]. He received abdominal radiotherapy for local node evolution in December 2018, and Nivolumab therapy was carried on for 68 cycles (December 2020) maintaining a partial response. It was stopped after local progression of the pre-cave lymph node and appearance of a new pancreatic lesion. Reported toxicity was adrenocortical insufficiency treated with hydrocortisone.

Circulating DNA ("liquid biopsy") was analyzed in January 2021, and pancreatic needle aspiration biopsy was performed in

February 2021, to prove melanoma progression, and to look for new mutations by NGS as mentioned in Fig. 1.

Retrospective analyses were performed on initial liver punch biopsy and all analyses reported the same *BRAF* non-V600 mutation N581K and NRAS mutation Q61L (Fig. 1). Main events of patient disease, and analyses performed are resumed on timeline (Fig. 2).

This class III *BRAF* mutation, leads to the inactivation of a kinase pathway, and treatment of which, when associated with *NRAS* mutation, is not codified. After a review of the literature, and genetic staff, MEKi (Trametinib) was initiated from March 19th, 2021 but patient died on April 10th, 2021, before the first clinical and radiological evaluation. BRAFi was not initiate because class III BRAF mutant are resistant to BRAFi, and this targeted therapy can over-activate MAPK pathway. This therapeutic reflection makes sense to initiate individual therapy, based on genetic analyses by NGS. Patient consent was obtained for all analyses.

At diagnosis, only IHC was performed, no BRAF V600 mutation was founded.

At Local and distant progression, NGS was performed on new metastatic lesion, and on first liver biopsy, retrospectively: the same BRAF type III mutation (N581K) and NRAS mutation (Q61L) were identified.

4. Discussion

In our case, we report a class III non-V600 BRAF mutation (BRAF N581K) with a *NRAS* mutation (*NRAS* Q61L). NGS results show that both mutants were present at the diagnostic and did not appear secondarily. The first line of treatment was immunotherapy because of a *BRAF non mutated V600*. No efficient second line treatment is available when progression occurs. Furthermore, data suggest a less favorable course of disease in patient harboring BRAF class II and III BRAF non V600 mutations [8,13].

When compared to other case reports from literature concerning type II and III non-V600 BRAF mutations: A 69 year-old man with stage IV melanoma, harboring class II mutation (*BRAF* K601E) [20], treated by MEKi alone (Trametinib) presented a partial response which lasted 2 months, before progression occurred. A 67-year-old man [20] with class III mutation (*BRAF* G466E) was treated by dacarbazine, and ipilimumab but rapidly died. A third case report [21], about a class II mutation (*BRAF* L597S) shows in a phase I study a partial response with the use of a new MEKi in monotherapy (MEK TAK 733); progression free survival was superior to 24 months [21]].

In 2017, NEMO study compared MEK inhibitors (binimetinib) to dacarbazine in stage III-IV *NRAS* mutated (Q61L; Q61K; Q61R) melanomas in first or second line (progression after immunotherapy). In this phase III study, PFS (progression free survival) was longer in the binimetinib group, but with no significant impact on OS (overall survival) [22]. One limitation of this study is, NRAS status was known, but only V600 BRAF mutations were screened and not any other BRAF exon 11 or exon 15. It's not surprising because at the time of the study, association between type III BRAF mutations and NRAS mutations was unknown and considered as mutually exclusive. However, recent molecular progress and NGS advent has undermined this credo. NRAS mutations are mutually exclusive with type I BRAF mutants, because the protein activity is enhanced, strong enough to activate the MAPK pathway and uncontrolled cell proliferation. This assertion is no longer valid for type II and III mutations.

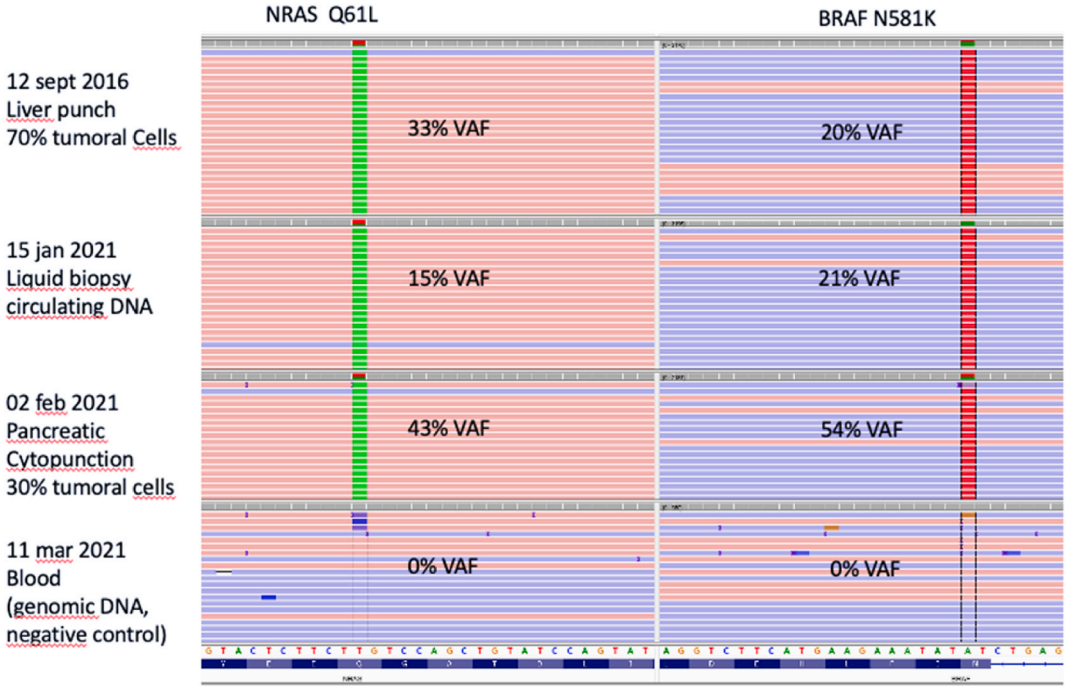

Fig. 1. Next generation sequencing result. As describe, BRAF N581K and NRAS Q61L mutations are presents in liver punch biopsy, pancreatic cytopunction, and liquid biopsy. VAF is the percentage of sequence reads matching the specific DNA variant.

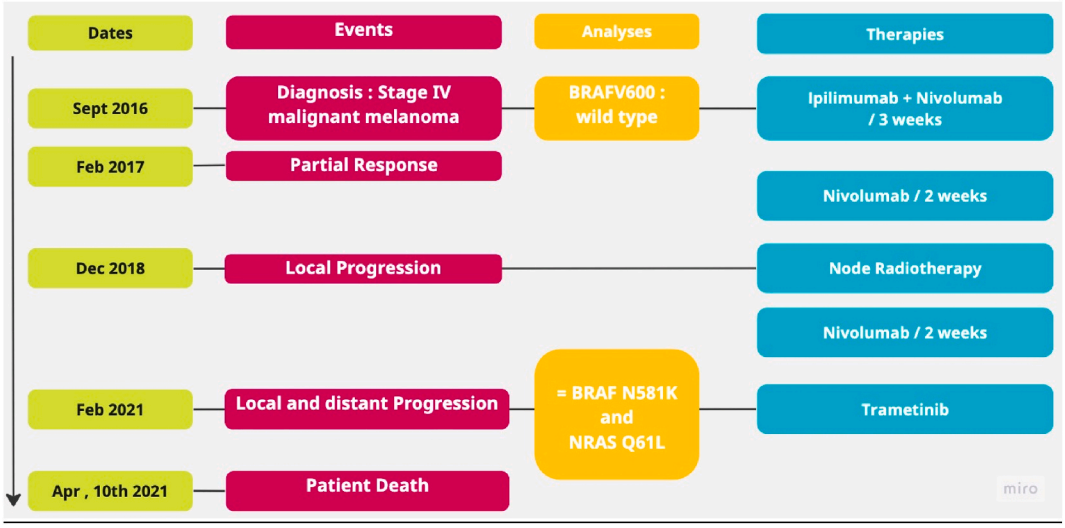

Fig. 2. Main events, therapies, and molecular analyses during patient history disease.

Type III BRAF mutations as cited in introduction correspond to low or dead kinase activity, and need, to activate proliferation and oncogenesis, to be associated to other mutations, like NRAS, to promote oncogenesis through others pathways.

Considering current knowledge and phase III studies, there is no study which aim to evaluate treatment efficiency in patients harboring both NRAS and non V600 BRAF mutation.

The study, "Targeted therapy in Advanced Melanoma with rare *BRAF* mutations "– 2019 [8] compared treatment with MEKi in monotherapy, monotherapy of BRAFi, and a combination of both in patients with V600 and non-V600 BRAF. Results did not show a significant difference between overall survivals in BRAFi + MEKi group *versus* MEKi group. It should be underlined that the number of patients in this study was poor. Also, *NRAS* status was unknown, and non-V600 BRAF mutations did not include the same mutation found in our patient.

In 2021, C.A. Nebhan et al. [23], tested in a phase II study, efficacy and safety of trametinib (MEKi), in stage III-IV BRAF non mutated V600 and BRAF fusion mutant melanoma, highlighted with NGS method. Patients were divided in two cohorts, differentiated by catalytic activity domain (cohort A: high/cohort B: low or unknown). Results showed a objective response rate (ORR) of 67% in cohort A, 17% in cohort B. The ORR was 0% for patient with class 3 mutation. This result suggested that MEKi should be proposed depending on the kinase activity.

To develop individual therapeutic approach, Patricia M. LoRusso et al. [24], conducted in 2015 a pilot study which enrolled five adult patients, metastatic or advanced unresecable metastatic melanoma patients, who were determined to be without a *BRAF V600* mutation. Treatment choice for a particular patient were recommended by the report of the tumoral genomic alterations, and the individualized treatment plan was based on the drugs knowledge and patient profile. One patient presented a *BRAF* class III mutation (D594G), two patients presented *NRAS Q61R* mutant, one *PTEN* and one *NF1*, The last was *CDKN2A* mutated, Four of five patients receive a MEKi (NRAS, BRAF, and NF1 mutated). The aim of this study was not to evaluate the efficiency of the target therapy, but the feasibility and promotion of their method based on sequencing, for individualized approach, called "precision medicine". Paula Martinez et al. [25] present their panel of new generation sequencing, OncoKitDx, which could be used in solid tumors with a specificity over 99,9% and a sensitivity of 100% with 5% limit of detection.

L.Boussemart et al., in 2018 [26], tested in the interest of using Hybrid Capture-Based Genomic profiling, to identify BRAF alterations initially negative by prior BRAF classical testing. They concluded that this method should be considered, particularly in patients with initial negative result with classical methods, because of the capacity to identify more alterations in one sample, allowing personal care.

To conclude, MEK inhibitor monotherapy was tested in second line for *NRAS* mutated metastatic melanoma with effective progression after first line immunotherapy, but no proof of OS increase has been reported. BRAF inhibitors cannot be used when *NRAS* is mutated, as they lead to over expression of the MAPK pathway through formation of BRAF-CRAF dimers [10]. In this way, a phase I study (NCT02437227) evaluated tolerance of a Pan RAF inhibitor, to stop dimers formation, retro-control and activation of alternative pathways. Another phase I/II study is ongoing "CHLORO TRAM MEL", evaluating tolerance of MEKi associated to plaquenil in metastatic, unresectable NRAS mutated melanoma.

We report a new association with rare non-V600 BRAF mutation that could be a target for new treatments in development.

There is an emergency to find new therapeutic options for this melanoma subtype, which appears to be more aggressive than *BRAF* wild-type melanomas [27]. Some *in vitro* studies are now evaluating news drugs targeting all classes of *BRAF* mutations (I, II, III), or drugs aiming to limit the tumor growth regardless of the *BRAF* status as lenvatinib.

We enjoin to carry out NGS in routine practice for all stage IV-MM, to identify a greater number of mutations, and thus develop therapies on new molecular targets rapidly. Secondly, we think that earlier identification of these alterations is essential to include

patients in clinical trials, necessary to speed up personalized therapeutic advances. Especially in our case, if the NGS had been done earlier, maybe patient could have benefited from a clinical trial.

New generation sequencing is a specific and sensitive molecular biology method to identify rare mutations. NGS seems to be essential, and should be performed for all patients, at diagnosis and sometimes to help identify a primary tumor but also during each event of disease progression, on each new metastatic target, to monitor the possible appearance of secondary mutants, during story disease, for individualized therapeutic approach in the future.

Indeed, the development of targeted therapies based on genetic mutations has now become a major axis in the management of patients, contrary to the conventional antiproliferative chemotherapies. In near future, we can therefore hope the democratization of genomic multidisciplinary meetings, first nationally, then worldwide, grouping cancers not on their histology subtype, as has long been the case, but on the basis of common genetic alterations.

Declarations

Author contribution statement

All authors listed have significantly contributed to the investigation, development and writing of this article. </p>

Data availability statement

Data included in article/supplementary material/referenced in article.

Additional information

No additional information is available for this paper.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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