**KRAS p.G12C mutation occurs in 1% of EGFR-mutated advanced non-small-cell lung cancer patients progressing on a first-line treatment with a tyrosine kinase inhibitor**

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**Background:** KRAS is mutated in ~30% of non-small-cell lung cancer (NSCLC) but it has also been identified as one of the mechanisms underlying resistance to tyrosine kinase inhibitors (TKIs) in EGFR-positive NSCLC patients. Novel KRAS inhibitors targeting KRAS p.G12C mutation have been developed recently with promising results. The proportion of EGFR-positive NSCLC tumours harbouring the KRAS p.G12C mutation upon disease progression is completely unexplored.

**Materials and methods:** Plasma samples from 512 EGFR-positive advanced NSCLC patients progressing on a first-line treatment with a TKI were collected. The presence of KRAS p.G12C mutation was assessed by digital PCR.

**Results:** Overall, KRAS p.G12C mutation was detected in 1.17% of the samples (n = 6). In two of these cases, we could confirm that the KRAS p.G12C mutation was not present in the pre-treatment plasma samples, supporting its role as an acquired resistance mutation. According to our data, KRASG12C patients showed similar clinicopathological characteristics to those of the rest of the study cohort and no statistically significant associations between any clinical features and the presence of the mutation were found. However, two out of six KRASG12C tumours harboured less common EGFR driver mutations (p.G719X/p.L861Q). All KRASG12C patients tested negative for the presence of p.T790M resistance mutation.

**Conclusions:** The KRAS p.G12C mutation is detected in 1% of EGFR-positive NSCLC patients who progress on a first line with a TKI. All KRASG12C patients were negative for the presence of the p.T790M resistance mutation.

**Key words:** KRAS, G12C, NSCLC, EGFR

**INTRODUCTION**

KRAS is the most frequently mutated oncogene in human cancers being mutated in ~30% of non-small-cell lung cancer (NSCLC).¹ It encodes a guanosine triphosphatase (GTPase) that in its active form [guanosine triphosphate (GTP)-bound] promotes cell proliferation. Mutated KRAS cannot return to the inactive guanosine diphosphate (GDP)-bound form leading to uncontrolled cell growth and proliferation.² NSCLC patients harbouring KRAS mutations constitute a heterogeneous group which have been associated to tobacco consumption and limited survival outcomes as well as resistance to EGFR tyrosine kinase inhibitors (TKIs).¹,³,⁴

For more than three decades, the development of targeted therapies against KRAS mutant tumours has been largely unsuccessful.¹⁵ Nevertheless, studies focusing on the potentially druggable KRAS p.G12C mutation have reported encouraging results.⁶,⁷ This mutation, which causes
the replacement of glycine by cysteine at 12 position, promotes active state of the KRAS protein triggering proliferation and it is found in 13% of lung adenocarcinomas being the most frequent variant in NSCLC. Specific KRAS p.G12C inhibitors are small molecules that bind irreversibly to the cysteine at residue 12, keeping KRAS at its inactive state.9

Nowadays, several direct KRAS<sup>G12C</sup> inhibitors have been developed and they are at different stages of clinical study. The first molecule developed AMG50 (sotorasib) has reported promising results from the phase I trial conducted in patients with heavily pre-treated advanced NSCLC harbouring the KRAS p.G12C mutation.10 In addition, a single-arm, phase II trial has recently reported a 37% response rate and 80% disease control rate in p.G12C-mutated advanced NSCLC previously treated with standard therapies.11,12 Similarly, the covalent MRTX849 has shown anti-tumour activity in cell line- and patient-derived xenograft models from different cancer types harbouring KRAS p.G12C mutation.12,13 Likewise, there are two novel inhibitors JNJ-74699157 and LY3499446 which are tested under phase I trials.

KRAS mutations have also been identified as an underlying mechanism of resistance to TKIs in EGFR-positive NSCLC.14 However, the role of KRAS inhibitors after treatment failure with a TKI in EGFR-positive NSCLC is completely unexplored.

The aim of this study is to assess the prevalence of KRAS p.G12C mutation after progression to a first-line TKI in EGFR-positive NSCLC patients with advance disease. To this aim, the presence of KRAS p.G12C mutation was tested in 512 plasma samples collected upon disease progression analysed by digital PCR (dPCR).

**MATERIALS AND METHODS**

**Patients and samples**

This is an observational study in which plasma samples from 512 NSCLC patients were analysed by dPCR. The study was approved by the ethical committee of Hospital Puerta de Hierro, Madrid, Spain (internal code: PIE14/0064 and PI 178-18) and was conducted in accordance with the precepts of the Code of Ethics of The World Medical Association (Declaration of Helsinki). Briefly, eligibility criteria included patients aged ≥18 years, with stage IV EGFR-positive NSCLC, who were progressing on a first-line treatment with a TKI. Samples from patients in whom progression was clinically suspected but not confirmed were also accepted. All patients provided the appropriate signed informed consent.

Between 2015 and 2019, 512 samples were collected upon disease progression to a TKI, in an 8.5-ml PPT<sup>™</sup> tubes (Becton Dickinson, Franklin Lakes, NJ). Plasma was isolated after two consecutive centrifugations. Specifically, samples were centrifuged at 1600 g for 10 min at room temperature followed by a second centrifugation round at 6000 g for 10 min. Circulating cell-free DNA (cfDNA) was isolated using a minimum starting volume of 3.5 ml of plasma and using the cfDNA QIAamp Circulating Nucleic Acid Kit (Qiagen®, Valencia, CA) following manufacturer’s protocol.

**dPCR analysis**

KRAS p.G12C mutation status was analysed by dPCR using predesigned TaqMan<sup>®</sup> dPCR assays in a QuantStudio® 3D Digital PCR (Applied Biosystems®, South San Francisco, CA). dPCR reaction was carried out in a final volume of 18 μl; this reaction included 8.55 μl of template cfDNA, 9 μl of 20X QuantStudio® Master Mix (ThermoFisher Scientific<sup>®</sup>, Palo Alto, CA) and 0.45 μl of 40X TaqMan assay (ThermoFisher Scientific<sup>®</sup>). Subsequently, 14.5 μl of final reaction volume was loaded to QuantStudio® 3D Digital PCR 20K Chip (ThermoFisher Scientific<sup>®</sup>). Thermal cycler conditions were: initial denaturation at 96°C for 10 min, 40 cycles at 56°C for 2 min, 98°C for 30 s and finally 60°C for 2 min and were maintained at 22°C for at least 30 min. Chips were read using QuantStudio® 3D Digital PCR instrument (ThermoFisher Scientific<sup>®</sup>). Results were analysed with QuantStudio® 3D AnalysisSuite<sup>™</sup> Cloud (ThermoFisher Scientific<sup>®</sup>). Default call assignments for each data cluster were manually adjusted when needed. Positive and negative controls were included in every run.

Mutant allele frequency (MAF) was defined as number of mutant molecules at a specific nucleotide location relative to the sum of total DNA molecules [mutant + wild type (wt)].

For sensitivity assays, DNA from a fresh tumour sample carrying the KRAS p.G12C mutation (as reported in the pathologist’s report) was mixed at different allele concentrations (i.e. 1%, 0.5%, 0.1% and 0.05%) with wt DNA extracted from peripheral blood cells from healthy donors. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation (SD) of the response and the slope according to International Conference on Harmonisation Q2 (R1) guideline. The SD of the response was calculated based on standard error of the y-intercept.

**Statistics**

Discrete variables are presented as frequencies and proportions, and continuous variables as means and SDs. Associations between KRAS p.G12C mutation status and clinicopathological variables were assessed using Fisher’s exact test or chi-square test according to which was most appropriate. The threshold of <i>P</i> < 0.05 was considered as statistically significant. Statistical software used was Stata v16.0 (StataCorp 2019, Stata Statistical Software Release 16, StataCorp LLC, College Station, TX). For survival analysis, median follow-up was estimated using reverse Kaplan—Meier method. Median overall survival (OS) and progression-free survival (PFS) were evaluated using Kaplan—Meier survival function. For OS analysis, time from the start of treatment with the first-line TKI to exitus or loss of follow-up was obtained, whereas for PFS, time was defined as the time from the start of treatment with the first-line TKI to disease progression, assessed by RECIST (Response Evaluation Criteria in Solid Tumours) criteria v1.1.
**RESULTS**

**Frequency of KRAS p.G12C mutation upon treatment failure with a TKI**

Clinical and epidemiologic characteristics of the 512 patients included in the study are presented in Table 1. The study population comprised mainly of females (64.45%) and never smokers (58.4%). The main histology was adenocarcinoma (93.36%). Regarding EGFR driver mutations, 90.74% were deletions in exon 19 or point mutations in exon 21 (56.77% and 33.97%, respectively). Mutations in exons 18 and 20 were also detected (3.56% and 5.23%, respectively). In two cases (0.48%), more than one driver mutation was detected. The p.T790M resistance mutation was present in 159 samples (31.83%).

The mean age at stage IV diagnosis was 66.17 years (192 patients with available data). First-line TKI was known for 193 patients with the following frequencies: 43.52% (n = 84) of the patients were treated with afatinib, 33.16% (n = 64) with gefitinib and 23.32% (n = 45) with erlotinib. Regarding metastases location at stage IV diagnosis, data were available for 190 patients. 50% (n = 95) of them showed local metastases, 31.05% (n = 59) had bone metastases, 18.42% (n = 35) presented metastases at central nervous system (CNS) and 13.16% (n = 25) showed liver metastases. Information about progression sites after first-line TKI treatment was available for 84 patients; among these patients, 63.10% (n = 53) presented progression disease at thoracic location and 26.19% (n = 22), 20.24% (n = 17) and 16.67% (n = 14) showed progression evidence at bone, CNS and liver, respectively. Finally, regarding second-line treatment, data were available for 99 patients, 51.52% (n = 51) of them received osimertinib, 21.21% (n = 21) were treated with first-/second-generation TKI, 18.18% (n = 18) received chemotherapy and 5.05% (n = 5), 3.03% (n = 3) and 1.01% (n = 1) received palliative care, immunotherapy and antiangiogenic agents, respectively.

The presence of the KRAS p.G12C mutation was evaluated in all samples. Only six samples (1.17%) were positive for this mutation (named as cases A-F) (Table 2) with an average MAF of 5.47% (SD: 8.08; min: 0.18%; max: 18.05%). In two cases (0.48%), more than one driver mutation was detected. The p.T790M resistance mutation was present in 159 samples (31.83%).

| Table 1. Clinicopathological characteristics of the study cohort according to KRAS p.G12C mutation |
|-------------------------------------------------|----------------|----------------|----------------|
| **Clinicopathological characteristics**         | **KRAS p.G12C** | **P value**    |
|                                                 | Non-mutated    | Mutated        |
| **Age, mean (SD), years**                       | 66.37 (10.99)  | 59.93 (7.66)   | 0.328          |
| **Sex, n (%) with data**                       |                |                |
| Female                                          | 325 (64.23)    | 5 (83.33)      | 0.430          |
| Male                                            | 181 (35.77)    | 1 (16.67)      |                |
| **Smoking, n (%) with data**                    |                |                |
| Never smoker                                    | 295 (58.30)    | 4 (66.67)      | 0.392          |
| Former smoker                                   | 174 (34.39)    | 1 (16.67)      |                |
| Active smoker                                   | 37 (7.31)      | 1 (16.67)      |                |
| **Histology, n (%) with data**                  |                |                |
| Adenocarcinoma                                  | 472 (93.28)    | 6 (100)        | 1.000          |
| Adenosquamous                                   | 17 (3.36)      | 0 (0)          |                |
| Large cell                                      | 7 (1.38)       | 0 (0)          |                |
| Undifferentiated                                | 9 (1.78)       | 0 (0)          |                |
| Other                                           | 1 (0.20)       | 0 (0)          |                |
| **First-line TKI, n (%) with data**             |                |                |
| Afatinib                                        | 81 (16.01)     | 3 (50)         | 1.000          |
| Erlotinib                                       | 44 (8.70)      | 1 (16.67)      |                |
| Gefitinib                                       | 62 (12.25)     | 2 (33.33)      |                |
| NA                                              | 319 (63.04)    | 0 (0)          |                |
| **Metastases location at stage IV, n (%) with data** |            |                |
| Local                                           | 94 (18.58)     | 1 (16.67)      | 0.621          |
| Bone                                            | 59 (11.66)     | 0 (0)          | 0.312          |
| CNS                                             | 34 (6.72)      | 1 (16.67)      | 0.560          |
| Liver                                           | 24 (4.74)      | 1 (16.67)      | 0.434          |
| NA                                              | 320 (63.24)    | 3 (50)         |                |
| **EGFR mutation, n (%) with data**              |                |                |
| Common                                          | 385 (76.09)    | 4 (66.67)      | 0.062          |
| Uncommon                                        | 28 (5.53)      | 2 (33.33)      |                |
| NA                                              | 93 (18.38)     | 0 (0)          |                |
| **EGFR p.T790M mutation, n (%) with data**      |                |                |
| Non-mutated                                     | 341 (67.39)    | 6 (100.00)     | 0.184          |
| Mutated                                         | 162 (32.02)    | 0 (0)          |                |
| NA                                              | 3 (0.59)       | 0 (0)          |                |
| **Second-line treatment, n (%) with data**      |                |                |
| First-/second-generation TKI                    | 21 (4.15)      | 0 (0)          | 0.007          |
| Antiangiogenic                                  | 1 (0.2)        | 0 (0)          |                |
| Immunotherapy                                   | 2 (0.4)        | 1 (16.67)      |                |
| Osimertinib                                     | 51 (10.08)     | 0 (0)          |                |
| Palliative care                                 | 5 (0.99)       | 0 (0)          |                |
| Chemotherapy                                    | 15 (2.96)      | 3 (50)         |                |
| NA                                              | 411 (81.23)    | 2 (33.33)      |                |
| **Progression site, n (%) with data**           |                |                |
| Local                                           | 52 (10.28)     | 1 (16.67)      | 0.552          |
| Bone                                            | 22 (4.35)      | 0 (0)          | 0.563          |
| CNS                                             | 16 (3.16)      | 1 (16.67)      | 0.497          |
| Liver                                           | 12 (2.37)      | 2 (33.33)      | 0.071          |
| NA                                              | 425 (83.99)    | 3 (50)         |                |

CNS, central nervous system; EGFR, epidermal growth factor receptor; NA, not available; SD, standard deviation; TKI, tyrosine kinase inhibitor.

**Assay performance**

Measured KRAS p.G12C MAFs correlated with their theoretical expected frequencies (Pearson’s correlation

immunotherapy. Finally, survival data were available for five of six patients, and the median follow-up for those patients was not reached (NR) (29.8-NR). The median PFS and OS were 18.5 months (95% CI: 5.2-NR) and 43.7 months (95% CI: 14-NR), respectively (Table 2).
coefficients 0.997). LOD and LOQ for KRAS p.G12C assays were 0.414% and 1.255% (Figure 1), respectively. LODs were estimated for samples with an average of 300 copies/ml of wt DNA. Additionally, 10 wt cfDNA from healthy donors were used to evaluate the false-positive signals. KRAS p.G12C mutation was not detected in any of the wt samples.

**DISCUSSION**

The development of targeted therapies against the KRAS p.G12C mutation is shifting the paradigm in the treatment of advanced NSCLC. However, this mutation is not assessed routinely in many clinical laboratories. Nevertheless, the identification of patients who might benefit from a KRAS inhibitor is crucial to plan treatment strategies. KRAS functions downstream of EGFR and it is a known mechanism underlying EGFR-TKI tumour resistance. It is well established that constitutive activation of KRAS, due to oncogenic mutations, activates EGFR pathway regardless of the EGFR status. Therefore, KRAS-mutated tumours are not expected to respond to EGFR blockade. MEK (mitogen-activated protein kinase kinase) is a downstream effector of the Ras GTPase encoded by KRAS and its activation plays a key role in intrinsic and acquired resistance to drugs targeting EGFR. In this way, co-targeting MEK and EGFR has been shown to overcome third-generation EGFR-TKI resistance. However, whether dual targeting of KRAS and EGFR may overcome drug resistance mutation or delay treatment failure in EGFR-positive NSCLC patients remains unknown. In this way, pre-clinical models suggest that blocking EGFR may reverse resistance to KRAS p.G12C. In this scenario, we believe that it is of clinical interest to determine how often the KRAS p.G12C mutation arose after treatment failure with a TKI in EGFR-positive NSCLC patients and whether targeting both mutations could improve survival in this subset of patients.

To our knowledge, this is the first study assessing the frequency of KRAS p.G12C mutation in EGFR-positive NSCLC patients upon disease progression. Noteworthy, recent studies focused on KRAS p.G12C mutation incidence exclude the EGFR-positive NSCLC population. Therefore, whether these patients could benefit from a KRAS inhibitor as a second-line treatment or concomitant to first-line EGFR-TKI is completely unexplored.

Overall, KRAS p.G12C is estimated to be mutated in 12% of all NSCLC. In our patient cohort, only 1.17% (n = 6) of EGFR-positive NSCLC tumours carried the KRAS p.G12C mutation upon disease progression. Of note, 512 samples were analysed. Unfortunately, we were unable to identify any clinical feature associated with the presence of the KRAS p.G12C mutation. Yet, the KRAS p.G12C mutation appears to be more frequent in younger patients (median age 60 versus 66 years) whose tumours harbour less common EGFR mutations such as the p.G719X or the p.L861Q.

Table 2. Clinical features of the six KRASG12C patients

| KRASG12C cases | A | B | C | D | E | F |
|----------------|---|---|---|---|---|---|
| Smoking status | Never smoker | Never smoker | Never smoker | Former smoker | Never smoker | Active smoker |
| Cigarettes/day | 10 | 10 | 10 | 10 | 10 | 10 |
| Sex | Female | Female | Male | Female | Female | Female |
| Previous cancer | No | NA | Testicle | NA | No | No |
| Age at diagnosis (years) | 63 | 67 | 48 | 58 | 56 | 68 |
| Histology | Adenocarcina | Adenocarcina | Adenocarcina | Adenocarcina | Adenocarcina | Adenocarcina |
| Metastasis location at stage IV diagnosis | Multiple brain metastases | NA | Liver | NA | NA | Extrathoracic lymph nodes | lung metastasis | adrenal glands |
| Diagnosis stage | IVB | IVA | IVB | IVA | IVA | IVA |
| EGFR mutation | ExDel19 | ExDel19 | G719X | ExDel19 | ExDel19 | L861Q |
| First-line TKI treatment | Gefitinib | Erlotinib | Afatinib | Gefitinib | Afatinib | Afatinib |
| First-line TKI start date | 16 March 2015 | 05 October 2012 | 27 June 2017 | NA | 04 February 2019 | 18 January 2018 |
| First-line progression | Yes | Yes | Yes | Yes | Yes | Yes |
| First-line progression date | 23 July 2018 | 23 January 2018 | 29 November 2017 | 09 November 2017 | 13 August 2020 | 04 January 2019 |
| Progression-free survival (months) | 40.8 | 64.5 | 5.2 | NE | 18.5 | 11.7 |
| Toxicity | No | NA | Diarrhoea | skin | NA | NA | Diarrhoea | skin |
| Radiotherapy | NA | NA | No | NA | NA | No |
| Second-line TKI treatment | Nivolumab | CT | CDDP + MTA | — | CT | — |
| Second-line TKI start date | 15 August 2018 | 15 March 2018 | 19 December 2017 | 24 August 2020 | — | — |
| Second-line progression | Yes | Yes | Yes | Yes | Yes | Yes |
| Second-line progression date | 17 October 2018 | 25 March 2019 | 27 March 2018 | 30 June 2021 | — | — |
| Progression site | Brain | NA | Liver | NA | NA | Liver | thoracic node | lung metastasis |
| Exitus date/last date follow-up | 17 October 2018 | 25 March 2019 | 20 August 2018 | 16 July 2018 | 29 April 2019 | — |
| Overall survival (months) | 43.7 | 78.7 | 14 | NE | 29.8 | 15.5 |

Adenocarcina, adenocarcinoma; CDDP, cisplatin; MTA, pemetrexed; NA, not available; NE, not estimated; CT, chemotherapy.
mutations. These results suggest that dual targeting of EGFR and KRAS might benefit a small proportion of patients (1% of EGFR-positive NSCLC patients). This information might be useful for sample size estimations in clinical trials addressing the efficacy of dual or consecutive EGFR and KRAS blockage. Remarkably, all KRAS G12C tumours tested negative for the presence for the p.T790M mutation. In any case, we cannot derive any solid conclusion given the small number of tumours with KRAS p.G12C mutation, but special attention will have to be paid to this population in future studies.

In our study, we did not have the pre-treatment formalin-fixed paraffin-embedded biopsy available for molecular analysis and we could only assess KRAS p.G12C status in the plasma sample of two of the six positive cases, which may constitute a limitation of our study. However, in both cases, the mutation was not detected, suggesting that this mutation arose as a resistance mechanism. In this regard, KRAS and EGFR mutations have been reported to occur very rarely simultaneously and their presence is believed to be mutually exclusive.23

In summary, our results indicate that KRAS p.G12C occurs in 1% of NSCLC patients progressing to a first-line TKI. Larger cohorts will be needed to identify clinical characteristics of the patients (if any) whose tumour progress through KRAS activation. Our results are of particular interest for the design of new clinical trials.

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