Research Paper

Rapid detection of EGFR mutations in decalcified lung cancer bone metastasis

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ABSTRACT

Detection of molecular alterations in lung cancer bone metastasis (LCBM) is particularly difficult when decalcification procedure is needed. The Idylla\textsuperscript{™} real-time (RT)-PCR is compared to the routine method used in our laboratory, which combines next generation and Sanger sequencing, for the detection of EGFR mutations in LCBM.

LCBM subjected to EDTA or formic acid decalcification were analysed for EGFR mutational status using two methods: first, the Ion Torrent Ampliseq next generation sequencing (NGS) assay +/- Sanger sequencing was used prospectively; then, the fully-automated, RT-PCR based molecular testing system Idylla\textsuperscript{™} EGFR Mutation Test was applied retrospectively.

Out of the 34 LCBM assayed, 14 (41.2%) were unsuitable for NGS analysis and five remained unsuitable after additional Sanger EGFR sequencing (5/34, 14.7%). Using Idylla\textsuperscript{™}, valid results were observed for 33/34 samples (97.1%). The concordance between the NGS +/- Sanger sequencing method and the RT-PCR method was 89.7% (26/29), one false positive EGFR S768I mutation and two false negative results were observed using Idylla\textsuperscript{™}; one of these false negative cases was diagnosed by Sanger sequencing with a rare exon 19 EGFR mutation not covered by the Idylla\textsuperscript{™} EGFR Mutation Test design.

Detection of EGFR mutations in decalcified LCBM is challenging using NGS, more than half of samples showing invalid results. Alternative methods should thus be preferred to spare clinical samples and decrease delay. The Idylla\textsuperscript{™} EGFR Mutation Test shows a good performance on decalcified bone samples and could be used as a first step. In case of negative results, a sequencing approach is mandatory to check the presence of rare EGFR mutations sensitive to EGFR tyrosine kinase inhibitors.

1. Introduction

Investigating molecular status and PD-L1 expression in lung non-squamous metastatic carcinoma allows personalised therapy to be proposed to patients [1]. Bone metastases are reported to occur in 30–50% of lung adenocarcinomas and may be synchronous in up to 15% of cases [2,3]. Patients may require metastasis biopsy when the primary pulmonary tumour is not accessible or when access to the primary lung tumour is precluded by co-morbidities. For these bone metastasis specimens, the decalcification process is a critical point. Nitric acid-based agents allow rapid tissue decalcification but lead to poor DNA quality and loss of antigenicity [4], preventing molecular and immunohistochemical analysis, respectively. Although next generation sequencing (NGS) techniques can generate interpretable results on
formalin-fixed paraffin-embedded (FFPE), EDTA and formic acid induce a higher degradation of DNA, and render molecular analysis challenging in routine practice [5,6].

In France, most molecular diagnostic interrogations of lung cancer samples depend primarily on NGS-based methods. In case of invalid NGS results, alternative methods to detect mutations are needed, introducing a delay in molecular status reporting. To avoid NGS failure and/or decrease delay in molecular status reporting, we theorized that targeted techniques could be used to diagnose EGFR mutations successfully and quickly [7]. The fully automated Idylla™ platform relies on real-time PCR (RT-PCR) technology to detect EGFR mutation. The test is performed directly on FFPE sections allowing a quick diagnosis of EGFR mutations. However this testing was developed for non-decalcified samples. In such conditions, the concordance between Idylla™ and other molecular techniques is high, ranging from 94% to 100% [8–12]. To our knowledge, no study has evaluated the performance of Idylla™ EGFR Mutation Test on decalcified lung cancer bone metastasis (LCBM).

The aim of this study was to compare the NGS +/- Sanger sequencing used in our department to the Idylla™ RT-PCR technology for the detection of EGFR mutation in LCBM subjected to decalcification procedure.

2. Material & methods

2.1. Patient and tumour samples

Tissue samples from patients with LCBM obtained between 2011 and 2018 in the pathology department of the Hospices Civils de Lyon (HCL), France were retrospectively collected. Only samples analysed following a decalcification procedure were included in the study. Clinical data collected were gender, age, type of sample and localisation of the tumour. Based on histopathological slides, lung carcinomas were classified according to the World Health Organization (WHO) histopathological classification [1]. All samples were included in the tumour bank “Tissu-tumorotheque Est” of the Biological Resource Centre (Centre de Ressource Biologique, CRB) of the HCL in Lyon, France and the present study was approved by the institutional ethics committee.

2.2. Decalcification procedure

For all LCBM, decalcification of bone was performed after formaldehyde fixation, using EDTA (0.5 M EDTA pH8.0, Promega, Madison, WI) from April 2016 to May 2017, then formic acid (Decalciﬁying Solution, Formic Acid 5%, Aqueous; Newcomersupply, Middleton, WI, USA) from June 2017 to December 2018. Decalcification was performed for a minimum of four hours for biopsies, and for four to 15 days for surgical specimens, using overnight alternate cycles (formaldehyde fixation / decalcification). EDTA and formic acid were chosen to increase preservation of cell morphology and DNA. Specimens were then dehydrated and paraffin-embedded according to routine procedures.

2.3. Analysis of genetic alterations using NGS +/- Sanger sequencing

Routine genetic alterations for oncogenic drivers (KRAS, EGFR, HER2, BRAF, MET exon 14) were detected prospectively. DNA was extracted from FFPE lung tumour tissue after laser capture micro-dissection (LCM) (Leica, LMD 6000, Wetzlar, Germany; QiAmp DNA micro-kit, QIAGEN, Venlo, Netherlands) which allowed to obtain tissue 2 mm², enriched in tumour cells. Molecular status was obtained by NGS using a laboratory developed ampisq panel and Ion Personal Genome Machine technology (PGM Ion Torrent, ThermoFisher Scientiﬁc, Waltham, MA, USA). In case of NGS failure due to poor DNA quality, EGFR Sanger sequencing was performed to obtain EGFR molecular status.

2.4. Idylla™ RT-PCR EGFR testing

Retrospectively, 4-μm FFPE sections obtained from the same samples as those subjected to the methods described above were subjected to the Idylla™ technology using the EGFR cartridges (Idylla™ platform, Biocartis, Mechelen, Belgium; Idylla™ EGFR Mutation Test, Biocartis). The specific mutations interrogated in this EGFR Mutation Test are described in the literature [9]. No micro- nor macro-dissection was performed for these analyses and the amount of tissue used varied from 20 to 600 mm². Percentage of tumour cells per section was determined. EGFR molecular status, type of mutation, and the cycle of quantification value (Cq) were collected.

3. Results

3.1. Patient and sample characteristics

A total of 76 bone metastatic non-squamous non-small cell lung carcinoma cases were collected between 2011 and 2018 in the pathology department of the HCL, amongst these 76 specimens, 34 underwent a decalcification procedure (44.7%). Patients were mainly men (24/34, 70.6%) with a median age of 70.0 years (range: 51–86). The specimens were obtained mainly from vertebralae (14/34, 41.2%) or hip (14/34, 41.2%), using CT-guided bone biopsies in 25 cases (73.5%). Most samples were adenocarcinomas (32/34, 94.1%). Decalcification was performed with EDTA in 26 cases (76.5%) or formic acid in eight cases (23.5%). Most samples (18/34, 52.9) contained more than 25% tumour cells while six samples (17.6%) contained less than 10% tumour cells (Table 1). Using either NGS +/- Sanger sequencing, KRAS mutations were detected in 11/34 cases (32.4%), EGFR mutations in 9/34 cases (26.5%; one case including a double EGFR mutation), and BRAF mutations in 2/34 cases (5.9%). In 20 cases (20/34, 58.8%), NGS failed and Sanger sequencing was required. Sanger sequencing-based analysis enabled to determine the molecular status of 15 additional samples. Finally, EGFR molecular status could not be determined for five samples (14.7%) due to low quality DNA and non-interpretable results by both NGS and Sanger sequencing (Table 2 and Fig. 1). Amongst the six cases containing less than 10% tumour cells, KRAS mutation was diagnosed in two cases and NGS +/- Sanger sequencing was unsuccessful in the four remaining cases.

Table 1

| Clinical and histopathological characteristics |
|-----------------------------------------------|
| Total population n = 34                       |
| Sex, n (%)                                    |
| male                                         |
| 24 (70.6)                                    |
| female                                       |
| 10 (29.4)                                    |
| Median age, years (range)                    |
| 70.0 (51–86)                                 |
| Tumour localisation, n (%)                   |
| hip                                          |
| 14 (41.2)                                    |
| vertebrae                                    |
| 14 (41.2)                                    |
| other                                        |
| 5 (17.6)                                     |
| Type of sample, n (%)                        |
| percutaneous biopsies                        |
| 25 (73.5)                                    |
| surgical samples                             |
| 9 (26.5)                                     |
| Histological type of NSCLC, n (%)            |
| Adenocarcinoma                               |
| 32 (94.1)                                    |
| NSCLC–NOS                                    |
| 2 (5.9)                                      |
| Decalcification procedure, n (%)             |
| EDTA                                         |
| 26 (76.5)                                    |
| Formic acid                                  |
| 8 (23.5)                                     |
| Percentage of tumour cells in the samples, n (%) |
| ≥ 25%                                        |
| 18 (52.9)                                    |
| 11–24%                                       |
| 10 (29.4)                                    |
| ≤ 10%                                        |
| 6 (17.6)                                     |

NSCLC–NOS: Non-small cell lung carcinoma – not otherwise specified.
3.2. Performance of the automated Idylla™ NGS = next generation sequencing; NI = non-interpretable result. Wild-type (WT) = no mutation diagnosed by sequencing for the EGFR (exon 19) gene.

Using the Idylla™ technology, valid results were obtained in 33 samples (33/34, 97.1%; Table 2 and Fig. 1) including four out of the five cases for which assessment by NGS +/- Sanger sequencing were unsuccessful (4/5, 80%). amongst them, EGFR mutations were detected in eight cases (8/33, 24.2%) including five exon 19 deletions (15.2%; one associated with T790M mutation), two L858R mutations (6.1%), and one S768I mutation (3.0%). The findings of the Idylla™ Mutation Test were in agreement with the reference method results in 26/29 cases (89.7%) with a negative percent agreement of 90.5% (19/21) and a positive percent agreement of 87.5% (7/8). amongst the three discordant cases, one case (S768I mutation) was diagnosed as KRAS G12C mutation by NGS +/- Sanger sequencing procedure and was considered as false EGFR positive result; re-test of this sample using a new cartridge of the Idylla™ EGFR Mutation Test confirmed the wild-type status for EGFR. amongst the 25 cases classified as wild-type for EGFR by Idylla™, two cases demonstrated EGFR mutations by NGS/ Sanger and were considered as false negative results; one case was a rare EGFR mutation (case 6; c.2252_2276delinsA; p.Thr751_Ile759delAsn) which was not covered by the design of the Idylla™ EGFR Mutation Test; the other false negative result (case 22) demonstrated a EGFR L858R mutation by NGS. These two false negative samples were decalcified with EDTA and contained between 11 and 25% of tumour cells. Finally, valid EGFR wild-type results were obtained for the six cases containing less than 10% of tumour cells.

4. Discussion

Despite the evolution in molecular techniques for detecting mutations in FFPE tissues, sequencing methods such as NGS or Sanger remain challenging in bone metastases samples subjected to decalcification. The performance of the Idylla™ EGFR Mutation Test was very good compared to NGS/Sanger for decalcified LCBM; most samples which were non-interpretable after NGS were interpreted using Idylla™. De Luca et al. had previously reported that out of the 37% (25/68) of samples unsuitable for NGS analysis, 80% were suitable with Idylla™ EGFR testing (20/25) [8]. In the present study however, NGS failure was higher, more than half of the samples being unsuitable. Since the NGS panel used in both studies was an amplicon panel, the difference could be explained by the bone decalcification procedure used herein, which alters DNA. Similarly to what was reported by De Luca et al. [8], the present study hence showed the superiority of the Idylla™ platform compared to that of NGS +/- Sanger sequencing. Moreover, while failure using NGS alone was higher in decalcified bone samples, the Idylla™ EGFR Mutation Test showed a similar performance in both non-decalcified [8] and decalcified samples. Furthermore, most of the samples included in this study were percutaneous biopsies, confirming the efficacy of the Idylla™ EGFR Mutation Test on small tissue areas [12]. Importantly, compared to NGS or Sanger, Idylla™ requires less manipulation and decreases delay for obtaining results [8–12].

In the literature, the concordance between Idylla™ EGFR Mutation Test and routine reference methods is very high, ranging from 94.0 to 100% on large series of sample [8–12]. Herein, the concordance in LCBM is lower, probably due to the smaller number of cases included.

### Table 2: Molecular characteristics of the samples.

| Patient N° | Decalcification procedure | Technique used for diagnosis | Molecular status by NGS +/- Sanger sequencing diagnosis | Cq by Idylla™ | Molecular status by Idylla™ |
|------------|---------------------------|-------------------------------|------------------------------------------------------|---------------|----------------------------|
| 1          | EDTA                      | Sanger                        | EGFR c.2325_2249del ; p.E745_A750del                  | 23.6          | Del Exon 19                |
| 2          | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 24.6          | S768I                      |
| 3          | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 19.9          | No EGFR mutation           |
| 4          | EDTA                      | NGS                            | EGFR c.2573T>G ; p.L858R                               | 21.4          | L858R                      |
| 5          | EDTA                      | Sanger                        | EGFR c.2252,2276delinsA ; p.T751_I759N                 | 18.2          | No EGFR mutation           |
| 6          | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 25.2          | Del Exon 19                |
| 7          | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 24.6          | S768I                      |
| 8          | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 24.6          | No EGFR mutation           |
| 9          | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 10         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 11         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 12         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 13         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 14         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 15         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 16         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 17         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 18         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 19         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 20         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 21         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 22         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 23         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 24         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 25         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 26         | EDTA                      | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 27         | Formic acid               | NGS                            | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 28         | Formic acid               | NGS                            | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 29         | Formic acid               | NGS                            | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 30         | Formic acid               | NGS                            | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 31         | Formic acid               | NGS                            | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 32         | Formic acid               | Sanger                        | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 33         | Formic acid               | NGS                            | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |
| 34         | Formic acid               | NGS                            | KRAS c.34G>T ; p.G12C                                  | 21.4          | L858R                      |

NGS = next generation sequencing; NI = non-interpretable result. Wild-type (WT) = no mutation diagnosed by sequencing for the KRAS (exon 2), EGFR (exon 18–21) and BRAF (exon 15) gene.
and the presence of one EGFR exon 19 deletion not covered by the Idylla™ design. Excluding this case, the concordance would have been higher than 90%. A KRAS positive sample determined by the reference NGS method was considered an EGFR S768I mutation by the Idylla™ platform. Performance of a second Idylla™ EGFR Mutation test demonstrated the EGFR wild-type status for this sample, which was thus considered a false positive. Similar S768I false positive results by Idylla™ have already been described in the literature [9] suggesting that caution should be applied when this specific mutation is reported by the Idylla™ EGFR Mutation Test. Because S768I EGFR mutation is a rare EGFR mutation, and its sensitivity to EGFR tyrosine kinase inhibitors (TKI) is still in debate [13–15], we suggest performing an alternative method before validating a positive report of this mutation. Alternatively, although the Idylla™ EGFR Mutation Test covers 51 EGFR mutations, some rare EGFR mutations that are sensitive to EGFR TKI are not covered by the test design. More precisely, according to our database as well as the literature [16], the c.2252_2276delinsA; p.(Thr751_Ile759delinsAsn) mutation not detected herein represents 0.2% of the EGFR mutations. In the present study, the patient whose LCBM harboured this rare EGFR mutation presented with multiple bone and cerebral metastases accompanying the lung mass. Under first generation EGFR TKI, the patient presented a partial response on lung and cerebral mass; unfortunately he died six months after diagnosis due to neurological complications. Although this mutation is rare, its rapid diagnosis is essential for clinical care and we think that if a second generation of Idylla™ EGFR Mutation Test is developed, it should include these rare exon 19 EGFR sensitive mutations.

The Idylla™ EGFR Mutation Test thus is a viable method to rapidly detect EGFR mutations in decalcified bone samples, showing a better performance than NGS. NGS testing is probably the best technique to optimise the use of precious small specimens and help patients find appropriate clinical trials. However, since the EGFR mutational status is what determines treatment approach in first line, EGFR testing should be adapted to specific cases, such as decalcified samples. Fig. 2 presents an algorithm that could be used for EGFR mutation detection in LCBM. Because NGS failure is high in decalcified samples, we suggest using RT-PCR for EGFR testing in first intention, followed by NGS in case of negative or invalid result.

CRediT authorship contribution statement

Antoine Boureille: Data curation, Investigation, Writing - original draft. Carole Ferraro-Peyret: Methodology, Writing - original draft, Supervision. Guillaume Pontarollo: Investigation, Data curation. Cyrille Confavreux: Investigation. Jean-Baptiste Pialat: Investigation. Sylvie Isaac: Investigation. Fabien Forest: Investigation. Violaone Yvorel: Investigation. Emmanuel Watkin: Investigation. Nicolas Girard: Conceptualization, Methodology, Writing - review & editing. Marie Brevet: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Writing - original draft, Writing - review & editing.

Conflict of Competing Interest

MB received grants from Biocartis, Astra Zeneca, BMS, and Pfizer to conduct research on lung cancer.
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