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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™ 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™ *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™, 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™; one of these false negative cases was diagnosed by Sanger sequencing with a rare exon 19 *EGFR* mutation not covered by the Idylla™ *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™ *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

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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 (Decalcifying 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 microdissection (LCM) (Leica, LMD 6000, Wetzlar, Germany; QIAmp DNA micro-kit, QIAGEN, Venlo, Netherlands) which allowed to obtain tissue 2 mm^2, enriched in tumour cells. Molecular status was obtained by NGS using a laboratory developed ampliseq 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 mm^2. 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 vertebrae (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.

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™ EGFR assay
1	EDTA	Sanger	*EGFR* c.2235_2249del ; p.E745_A750del	23.6	Del Exon 19
2	EDTA	Sanger	*KRAS* c.34G>*T* ; p.G12C	24.6	S768I
3	EDTA	NGS and Sanger both NI	NI	27.4	No EGFR mutation
4	EDTA	NGS	*KRAS* c.34G>*T* ; p.G12C	19.9	No EGFR mutation
5	EDTA	NGS	*EGFR* c.2573T>*G* ; p.L858R	21.4	L858R
6	EDTA	Sanger	*EGFR* c.2252_2276delinsA ; p.T751_I759N	18.2	No EGFR mutation
7	EDTA	Sanger	*EGFR* c.2239_2256del p.L747_S752del	25.2	Del Exon 19
8	EDTA	NGS	*KRAS* c.35G>*A* ; p.G12D	22.9	EGFR WT
9	EDTA	NGS	*EGFR* c.2235_2249del ; p.E745_A750del + *EGFR* c.2369C>*T* ; T790M	22.7	Del Exon 19 + T790M
10	EDTA	NGS and Sanger both NI	NI	26.2	No EGFR mutation
11	EDTA	Sanger	No *KRAS, EGFR, BRAF* mutation	30.9	No EGFR mutation
12	EDTA	Sanger	No *KRAS, EGFR, BRAF* mutation	24.6	No EGFR mutation
13	EDTA	Sanger	*BRAF* c.1780 *G* > *A* ; p.D594N	23.1	No EGFR mutation
14	EDTA	NGS	*KRAS* c.34_35delinsTT ; p.G12F	20.9	No EGFR mutation
15	EDTA	Sanger	*KRAS* c.34G>*T* ; p.G12C	21.5	No EGFR mutation
16	EDTA	NGS	No *KRAS, EGFR, BRAF* mutation	21.9	No EGFR mutation
17	EDTA	NGS	No *KRAS, EGFR, BRAF* mutation	21.2	No EGFR mutation
18	EDTA	NGS	No *KRAS, EGFR, BRAF* mutation	20.7	No EGFR mutation
19	EDTA	NGS	*KRAS* c.35G>*T* ; p.G12V	21.0	No EGFR mutation
20	EDTA	NGS	No *KRAS, EGFR, BRAF* mutation	20.9	No EGFR mutation
21	EDTA	NGS	*BRAF* c.1799T>*A* ; p.V600E	24.0	No EGFR mutation
22	EDTA	Sanger	*EGFR* c.2573T>*G* ; p.L858R	27.6	No EGFR mutation
23	EDTA	NGS	*EGFR* c.2240_2254del ; p.Leu747_Thr751del	22.4	Del Exon 19
24	EDTA	Sanger	*KRAS* c.35G>*A* ; p.G12D	21.7	No EGFR mutation
25	EDTA	Sanger	*KRAS* c.34G>*T* ; p.G12C	22.0	No EGFR mutation
26	EDTA	Sanger	*EGFR* c.2573T>*G* ; p.L858R	25.1	L858R
27	Formic acid	NGS and Sanger both NI	NI	22.1	No EGFR mutation
28	Formic acid	NGS	*KRAS* c.35G>*T* ; p.G12V	19.7	No EGFR mutation
29	Formic acid	Sanger	*KRAS* c.34G>*T* ; p.G12C	22.2	No EGFR mutation
30	Formic acid	Sanger	*KRAS* c.35G>*A* ; p.G12D	22.3	No EGFR mutation
31	Formic acid	NGS	*EGFR* c.2236_2250del ; p.E746_A750del	22.4	Del Exon 19
32	Formic acid	Sanger	No *KRAS, EGFR, BRAF* mutation	22.1	No EGFR mutation
33	Formic acid	NGS and Sanger both NI	NI	21.0	No EGFR mutation
34	Formic acid	NGS and Sanger both NI	NI	NI	NI

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.

3.2. Performance of the automated Idylla™ RT-PCR assay for the diagnosis of EGFR mutations in decalcified LCBM

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™ *EGFR* 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 a 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_Ile759delinsAsn) 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 for 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

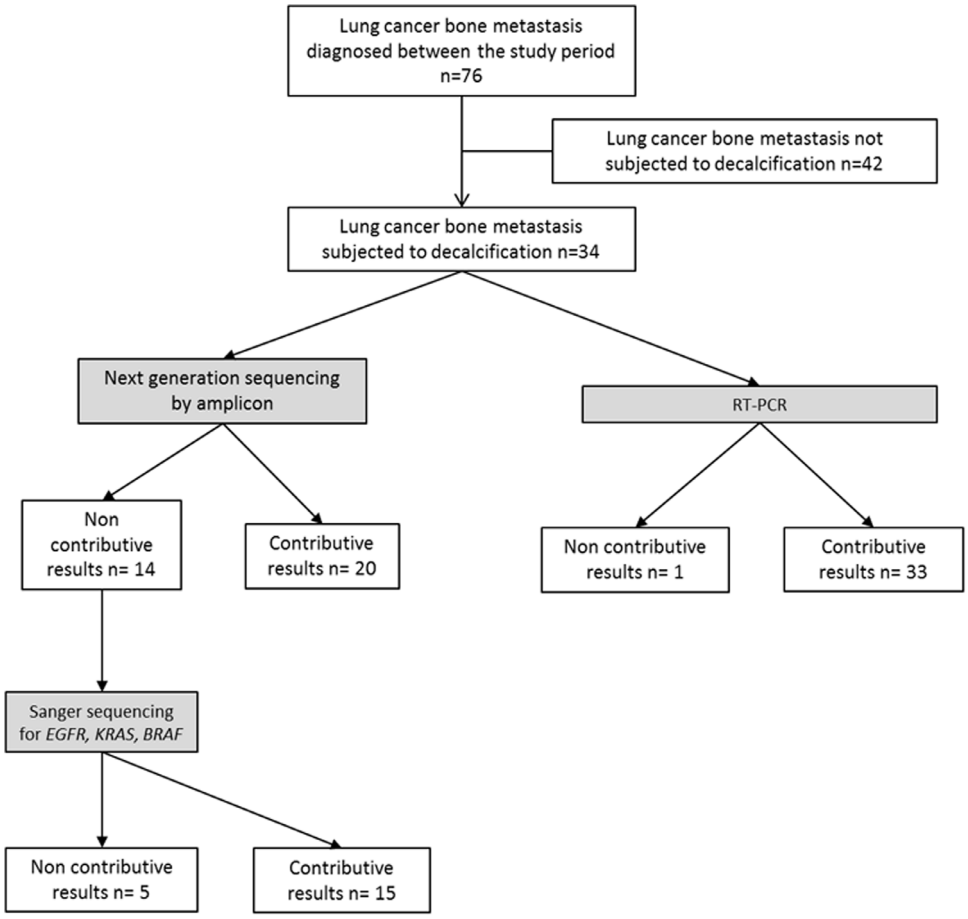

Fig. 1. Performance of next generation sequencing +/- Sanger sequencing versus the Idylla™ real time (RT)-PCR for the detection of *EGFR* mutation.

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. **Violaine 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.

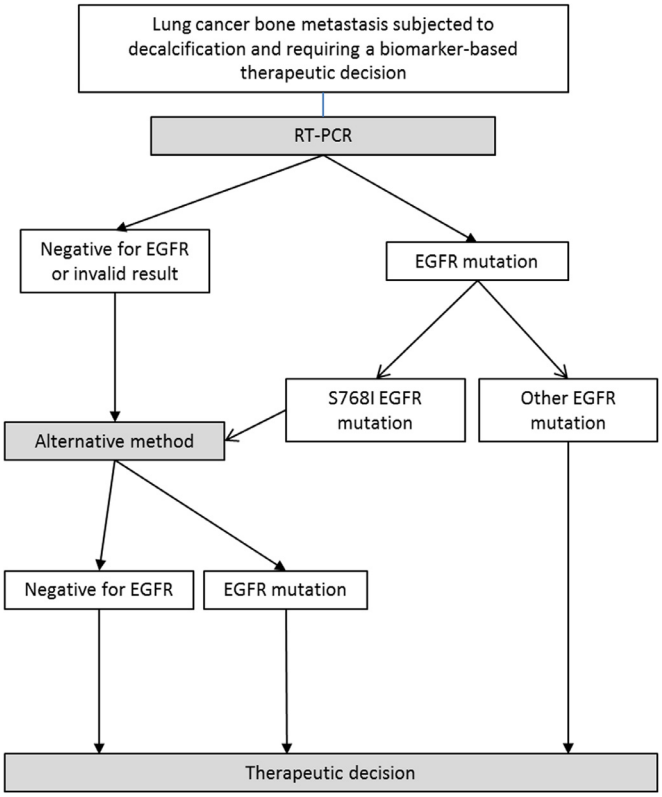

Fig. 2. Proposed decisional algorithm for *EGFR* mutation detection in lung cancer bone metastasis using real time (RT)-PCR.

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