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Molecular profiling of non-small-cell lung cancer patients with or without brain metastases included in the randomized SAFIR02-LUNG trial and association with intracranial outcome

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ABSTRACT

Introduction: Lung cancer remains the most frequent cause of brain metastases (BMs) and is responsible for high morbidity and mortality. Intracranial response to systemic treatments is inconsistent due to several mechanisms: genomic heterogeneity, blood–tumor barrier, and the brain-specific microenvironment. We conducted a study using data from the SAFIR02-LUNG trial. The primary objective was to compare the molecular profiles of non-small-cell lung cancer (NSCLC) with or without BMs. The secondary objective was to explore central nervous system (CNS) outcomes with various maintenance treatment regimens.

Methods: In total, 365 patients harboring interpretable molecular data were included in this analysis. Clinical and biological data were collected. Genomic analyses were based on array-comparative genomic hybridization and next-generation sequencing (NGS) following the trial recommendations.

Results: Baseline genomic analyses of copy number variations identified a 24-gene signature specific to lung cancer BM occurrence, all previously known to take part in oncogenesis. NGS analysis identified a higher proportion of *KRAS* mutations in the BM-positive group (44.3% versus 32.3%), especially G12C mutations (63%

Abbreviations: ANSM, *agence nationale de sécurité du medicament*; BBB, blood brain barrier; BM, brain metastasis; CGH, comparative genomic hybridization; CI, confidence interval; CNS, central nervous system; CPP, *comité de protection des personnes*; CT scan, computer tomography; DNA, deoxyribonucleic acid; ECOG, Eastern Cooperative Oncology Group; FFPE, formalin-fixed paraffin-embedded; ICI, immune checkpoint inhibitor; MRI, magnetic resonance imaging; MTB, molecular tumor board; NGS, next generation sequencing; NMDA, N-methyl-D-aspartate; NSCLC, non-small-cell lung cancer; OR, Odds ratio; OS, overall survival; PCR, polymerase chain reaction; PDL1, programmed death ligand 1; RECIST, response evaluation criteria in solid tumors; RNA, ribonucleic acid; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; TFR1, transferrin receptor protein 1; VEGF, vascular endothelial growth factor.

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versus 47%). Protein interaction analyses highlighted several functional interactions centered on EGFR. Furthermore, the risk of CNS progression was decreased with standard pemetrexed maintenance therapy. The highest rate of CNS progression was observed with durvalumab, probably because of the specific intracranial immune microenvironment.
Conclusion: This work identified a 24-gene signature specific to lung cancer with BM. Further studies are needed to precisely determine the functional implications of these genes to identify new therapeutic targets for the treatment of lung cancer with BM.

1. Introduction

Metastatic non-small-cell lung cancer (NSCLC) remains the leading cause of cancer-related death worldwide [1,2], particularly because of the high incidence of brain metastases (BMs) [3]. Lung cancer is indeed the most frequent cause of BM, with almost 50% of BM cases originating from lung cancer; BM is present in approximately 10% of NSCLC patients at initial diagnosis and in approximately 50% of patients during the entire course of the disease [4]. Despite significant improvements in local and systemic treatments during the past decade, NSCLC BMs are still responsible for high morbidity and mortality, with median overall survival (OS) not exceeding 12 months [5]. Moreover, local treatment toxicity affects the quality of life, and this has to be considered [6]. Thus, there is a need to enhance our knowledge of BM biology to improve NSCLC patient outcomes.

Even though the cellular mechanisms underlying metastatic dissemination may be well understood, the genomic and phylogenetic characteristics of brain invasion mostly remain to be elucidated. The molecular dissection of lung cancer revealed that some mutations were associated with a high (*EGFR*, *ALK*) [7] or low (*KRAS*)[8] incidence of BM, providing a glimpse into the molecular features of the central nervous system (CNS) metastatic process. Although the numbers are limited, published molecular data on BMs have highlighted significant genomic heterogeneity in BMs compared with primary tumors [9] or other metastatic sites. For example, Jiang *et al.* [10] recently reported significantly higher genomic heterogeneity between primary tumors and BMs (median 6.8% of shared mutations) than between primary tumors and liver metastases (median 66.3% of shared mutations; $p = 0.005$). Moreover, phylogenetic reconstructions based on these data suggest that the brain metastatic process occurs earlier than other distant lesions. These particular genomic features of BMs were confirmed and expanded in a study recently published by our team, which successfully identified recurrent mutations in 13 genes (*AFF2, ANO3, CCDC178, CRISP3, DRD5, FAM134A, LOR, NCOR2, NELFB, MUC2, RUNX1T1, SPATA31C1,* and *TENM3*) never previously identified in primary tumor samples [11]. Even though the blood–brain barrier (BBB) is certainly involved in this molecular divergence [12], taken together, these data support the scenario that a common precursor for lung primary tumors, BMs, and other distant lesions exist before they start evolving on their own account. During this evolution, the number of mutations and chromosome alterations acquired is higher in BMs than in primary tumors or other metastases [10]. However, interactions between BM molecular profiles and systemic drug efficiency have so far been poorly explored.

The CNS outcome of patients undergoing systemic treatments is still largely unpredictable because of the impact of the BBB on the pharmacokinetics and pharmacodynamics of cancer therapeutics [13]. Therefore, morbidity and mortality from BMs are aggravated by the frequent discrepancies between intracranial and extracranial responses to systemic treatments. In NSCLC, adding an anti-angiogenic drug – anti-vascular endothelial growth factor (VEGF) monoclonal antibody, bevacizumab – to standard platinum-based chemotherapy was the first regimen to suggest an improvement in BM outcome [5]. Subsequently, despite a higher incidence of BMs among *EGFR*-mutant or *ALK*-rearranged NSCLC, impressive CNS efficacy of new generations of tyrosine kinase inhibitors, especially osimertinib and alectinib, successfully turned BM patients into long responders [14,15]. The CNS outcome in patients under immune checkpoint inhibitors (ICIs) is widely variable, mainly because of the exclusion of patients from clinical trials, a frequent use of systemic corticosteroids [16], and because of the unique immune environment in the CNS (such as the lack of cytotoxic T lymphocytes or the functional implications of astrocytes) [17].

Considering these characteristics, a better knowledge of the biology of lung cancer BM will be crucial to improve lung cancer outcomes. Given the technical limits of performing molecular analysis on BM samples in daily care, the identification of a BM-specific genomic signature among primary lung tumors or distant metastases will help to improve the management of these patients. Moreover, increased knowledge of CNS outcome in patients under various NSCLC treatment regimens (chemotherapy, targeted therapy, ICIs) will be crucial for BM management. The current study was based on data from the randomized SAFIR02-LUNG trial [18] (Essai intergroupe Unicancer 0105–1305/ IFCT1301 SAFIR02-LUNG – NCT02117167), including exhaustive clinical and molecular data on metastatic lung cancer patients, as well as clinical outcomes under various systemic first-line maintenance treatment regimens (chemotherapy, ICI, targeted therapies). The primary objective of this translational study was to compare the DNA mutational and copy-number profiles of NSCLC with or without BMs to establish a molecular signature associated with BMs. The secondary objective was to evaluate CNS outcome on various maintenance treatment regimens based on baseline BM status.

2. Materials and methods

2.1. SAFIR02-LUNG trial

The French multicenter randomized phase II SAFIR02-LUNG trial enrolled 999 treatment-naïve patients with advanced NSCLC. Other main inclusion criteria were the absence of *EGFR*- or *ALK*-activating alterations, the availability of tissue samples from primary tumors or metastases (excluding bone) suitable for molecular analysis (cell-free DNA molecular data from the SAFIR02-LUNG trial were excluded in our study), and eligibility for a first-line platinum-based regimen. Patients had to show objective response or stable disease after a platinum-based regimen according to the response evaluation criteria in solid tumors (RECIST) 1.1. Exclusion criteria could be summarized as all the potential contraindications to one of the study drugs (Supplementary Table 1 reports the details of eligibility criteria). Regarding CNS status, only the presence of symptomatic or progressive untreated BMs after induction chemotherapy was considered an exclusion criterion. According to the study form, baseline brain MRI or CT scan was required for every patient at enrollment. During follow-up, iterative brain imaging (CT scan or MRI) was required for baseline BM-positive patients or in cases of neurological symptoms.

This open-label trial used high-throughput genome analysis as a therapeutic decision tool to compare experimental (arm A) versus standard (arm B) maintenance treatments, targeted treatment (Supplementary Table 2) versus standard treatment if a targetable alteration was identified (substudy 1, 175 randomized patients), or durvalumab (anti-programmed death ligand 1, PDL1) immunotherapy versus standard treatment in the absence of targetable molecular alteration or contraindication to targeted therapies (substudy 2, 183 randomized patients). The standard maintenance therapy arm was based on

pemetrexed for non-squamous NSCLC or erlotinib for squamous NSCLC. Substudy 1 eligibility was defined by the presence of a targetable molecular alteration according to the molecular tumor board (MTB), and molecular alterations were classified into four categories (Supplementary Table 2). Otherwise, patients were eligible for randomization in substudy 2. The randomization was based on a 2:1 ratio in favor of the experimental arm in each substudy.

2.2. Study population

Patients were selected from the SAFIR02-LUNG trial, and our study therefore followed the trial inclusion and exclusion criteria (Supplementary Table 1). For our first objective, every screened patient was eligible, and every randomized patient was eligible for our secondary objectives, on the condition of the availability of qualitative molecular data.

BM status at baseline was collected from the trial case report form, and CNS outcome was assessed by investigators based on scheduled tumor assessment brain CT scan or MRI (systematic in the case of baseline BMs or neurological symptoms). Disease progression was defined by the appearance of a new brain lesion, regardless of baseline BM status.

2.3. Genomic analysis

Archival biopsy or new biopsy before cycle 3 of the induction chemotherapy was required for screening and sent to one of the five participating genomic platforms (Gustave Roussy, Centre Leon Bérard, Institut Curie, Institut Bergonié, and Institut de Cancérologie de l'Ouest) for DNA extraction, quality control, and genomic analysis. Array-comparative genomic hybridization (array-CGH) and next-generation sequencing (NGS) were performed. Molecular analysis was performed if tumor cellularity was $>$ 30% for frozen samples and $>$ 10% for formalin-fixed paraffin-embedded (FFPE) samples. For FFPE tissue sections (six sections of 6 μm each), tumor-rich areas were macro-dissected, and the samples were digested with proteinase K before DNA extraction with the Maxwell RSC DNA FFPE kit (Promega) kit or the QIAmp DNA FFPE tissue (Qiagen). DNA extraction from frozen biopsies was performed using the AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's protocol. DNA was quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific). In the absence of tissue DNA, circulating tumor DNA was used for molecular analysis and extracted using QIAamp Circulating Nucleic Acid (Qiagen).

The NGS panel included 65 critical oncogenes or tumor suppressor genes. The initial PCR step was performed using a 10-ng DNA sample, and amplicons were partially digested to remove primer sequences before ligation with adapters and barcodes, amplification and purification. After quality and quantity assessment, libraries were pooled at equimolar ratios and sequenced using IonTorrentS5 or Illumina MiSeq technologies. Array-CGH analysis was performed on the Affymetrix platform with the CytoScan HD array Kit for DNA extracted from frozen samples and the OncoScan FFPE Assay Kit for DNA extracted from FFPE tissues. Both technologies used single-nucleotide polymorphism probes to provide DNA copy number variations. Genomic data were systematically analyzed by the MTB to confirm the pathogenicity of identified variants and thus patient eligibility for substudy 1.

2.4. Bioinformatic analysis

For NGS analysis, all variants passing the following thresholds were validated: depth of coverage $>$ 100X, allelic ratio $>$ 5%, and population frequency $<$ 0.1% in either 1000 g, ESP or gnomAD. All somatic mutations were annotated, sorted, and interpreted by an expert molecular biologist according to available databases (Cosmic, TumorPortal, TCGA, Cancer Hotspots, etc.). Pathogenic variants were defined as follows: (i) for oncogenes, only mutations driving gain of function were considered (i.e., hotspot missense mutations, in-frame insertions/deletions described as oncogenic in the literature), (ii) for tumor suppressor genes (TSGs), only mutations leading to loss of function were considered: that is, truncating alterations (nonsense mutations, frameshift insertions/deletions/splicing) or missense mutations described as deleterious in the literature.

Regarding array-CGH analysis, copy number variations from CytoScan and OncoScan were defined using the R package rCGH (v1.16.0 under R v3.6.3). Log_2 relative ratios were calculated before centralization of the profile to set the baseline from which copy number alterations were estimated (two copies being the neutral level). Break points in the log_2 relative ratio continuity were identified by profile segmentation. These segments were used to detect focal gene amplifications (log_2 ratio $>$ 1.58, i.e., fold change $>$ 6 DNA copies, and amplicon size $<$ 10 Mb) or homozygous deletions (log_2 ratio $<$ –1) and were discussed during the tumor board meeting. Focal amplifications and homozygous deletions were compared among the predefined groups, and a p-value $<$ 0.05 before adjustment was considered statistically significant. Oncogenic driver evaluation among regions of interest relied on two different approaches. First, the candidate strategy presumed the oncogenic driver to have been described previously as a somatic alteration in lung cancer (among a 23-gene selection) [19]. Second, every potential driver was assessed by the discovery strategy, which consisted of a screen among oncoKB and PubMed databases. Array-CGH analysis was focused on focal amplification and homozygous deletions because of their theragnostic impact.

2.5. Statistical analysis

Data were summarized by frequency and percentage for categorical variables and by median and range for continuous variables. Comparisons between groups were performed using the Wilcoxon rank sum test for continuous variables and chi-square or Fisher exact test for categorical variables. All statistical tests were two-sided, and differences were considered statistically significant when p $<$ 0.05. A series of multivariate logistic regression models were used with *KRAS* mutation as the response variable and patient's baseline characteristics as predictor variables. To investigate the potential factors associated with the over-representation of *KRAS* mutation among BM-positive patients, an interaction term between each variable and the BM status was added to each model one by one. R version 3.6.3 was used for the statistical analyses.

2.6. Ethical framework

The SAFIR02-LUNG trial was approved by the French ethics committee CPP Ile de France 2 on 09 November 2013 (2013–08-04) and the French health authorities ANSM on 17 October 2013 (130975A-12). The SAFIR02-LUNG study was conducted in accordance with the Declaration of Helsinki, current International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines, and all applicable regulatory and ethical requirements. Patients signed informed consent for biopsy, randomization, and use of their biological samples for research purposes. Concerning our study, all patients signed informed consent for the SAFIR02-LUNG trial and ancillary studies, and a license contract was established to frame the SAFIR02-LUNG trial data utilization by the investigators (RCAPHM14 0097).

3. Results

3.1. Population

Patients were enrolled between April 2014 and December 2018 at 37 centers in France. Among the 999 enrolled patients, CNS status at baseline was available for 785 patients, and 365 had sufficient genomic

data quality to be included. Array-CGH data were collected from all 365 patients, and NGS data were collected from 360 of them (five were excluded because of insufficient tumor tissue sample quality). The population was first divided into two groups according to the baseline BM status, with 107 patients harboring BMs, including 67 patients with multiple brain lesions (two or more). Among the 107 BM-positive patients, 47 (44%) benefited from initial local brain radiation therapy. Patient characteristics, summarized in Table 1, highlighted that a majority of the patients in the BM-positive group were active smokers (58.9% versus 44.2%; $p < 0.05$).

3.2. Baseline array-CGH analysis

The most frequent focal amplifications were in 5p15.33 (*TERT*), 8q24.21 (*MYC*), and 14q13.3 (*NKX2-1*) in both the BM-positive and BM-negative groups. The candidate strategy failed to identify focal amplifications significantly associated with BM status. With the discovery strategy, significant focal amplification variation was identified among 12 genes (and 12 adjacent regions) comparing the BM-positive and BM-negative populations (Table 2, Fig. 1a). Except for *EGLN3* (and adjacent *NFKBIA*), focal amplification always favored the BM-positive group. Focal amplification of the *BCL2L1* gene is illustrated in Fig. 1b. These 12 genes have already been described as part of the oncogenic process. However, neither candidate nor discovery strategy succeeded in identifying a significant variation in homozygous deletion between the BM-positive and BM-negative populations.

3.3. Protein interaction analyses

To explore the functional implications of our findings, we performed protein interaction analyses using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) molecular database. Among the 12 identified genes, only BCL2L1 and TNS1 proteins were found to interact directly (supported by co-expression, experimental data, and literature data). When the 12 adjacent gene products were added to the process, several interactions were identified (Fig. 2a), centered on EGFR, but 13 gene products didn't show any interaction and were removed from the illustration. Finally, protein interactions were assessed between the 12 genes identified in our study and the 13-gene signature previously published by our team [11]. As shown in Fig. 2b, additional protein interactions were observed with RUNX1T1, NCOR2, and LOR among the 13 genes identified in the pilot study.

Table 1
Baseline patients and tumor characteristics according to brain metastasis (BM) status.

Characteristic	BM-positive group (n = 107)	BM-negative group (n = 258)	*p-value*
Age (range)	64.1 (33.0–80.9)	58.9 (32.7–83.8)	*0.116*
Sex			
Male	60 (56.1%)	167 (64.7%)	*0.152*
Female	47 (43.9%)	91 (35.3%)	
			<0.05
Smoker status	63 (58.9%)	114 (44.2%)	
Active smoker	31 (29.0%)	119 (46.1%)	
Former smoker	11 (10.2%)	23 (8.9%)	
Never smoker	2 (1.9%)	2 (0.8%)	
Data missing			
Histology	107 (100%)	258 (100%)	
Non-squamous-cell carcinoma			
Squamous-cell carcinoma	0 (0%)	0 (0%)	
Initial stage			
I	2 (1.9%)	2 (0.8%)	*0.053*
II	0 (0%)	1 (0.4%)	
III	0 (0%)	15 (5.8%)	
IV	105 (98.1%)	240 (93.0%)	
Median number of metastatic sites	4 (1–9)	3 (1–9)	*<0.05*
Randomized proportion	47 (43.9%)	106 (41.1%)	
Substudy 1	32 (68.1%)	73 (68.9%)	*1*
Substudy 2	15 (31.9%)	33 (31.1%)	
First-line treatment			*0.06*
Platin/pemetrexed	95 (88.8%)	223 (86.5%)	
Platin/paclitaxel	4 (3.7%)	23 (8.9%)	
Platin/vinorelbine	1 (0.9%)	0 (0%)	
Platin/gemcitabine	0 (0%)	4 (1.5%)	
Other	7 (6.6%)	8 (3.1%)	

Table 2
Focal amplifications significantly associated with brain metastasis (BM) status.

Chr†	FA†† samples (%)		Gene	Adjacent gene(s)	*p-value*
	BM-positive group (n = 107)	BM-negative group (n = 258)			
2q35	3 (2.80%)	0 (0%)	*TNS1*		*0.039*
3q29	4 (3.73%)	0 (0%)	*PAK2*	*TFRC*	*0.010*
7p12.1	9 (8.41%)	7 (2.71%)	*COBL*	*EGFR*	*0.032*
7q36.1	8 (7.48%)	6 (2.32%)	*CDK5*	*AGAP3*	*0.042*
10q26.3	6 (5.61%)	2 (0.77%)	*GLRX3*	*MKI67*	*0.013*
12p13.33	6 (5.61%)	2 (0.77%)	*WNT5B*	*RAD52*	*0.013*
12q24.31	3 (2.80%)	0 (0%)	*SETD1B*		*0.039*
14q13.1	0 (0%)	14 (5.43%)	*EGLN3*	*NFKBIA*	*0.031*
16q12.1	4 (3.74%)	1 (0.39%)	*TOX3*		*0.044*
19q13.2	8 (7.48%)	4 (1.55%)	*RYR1*	*MAP4K1/ ACTN4*	*0.010*
19q13.31	6 (5.61%)	3 (1.16%)	*PVR*	*CEACAM19/ BCL3*	*0.034*
20q11.21	6 (5.61%)	3 (1.16%)	*BCL2L1*	*TPX2/FGR1B*	*0.034*

† Chromosome.
†† Focal amplification.

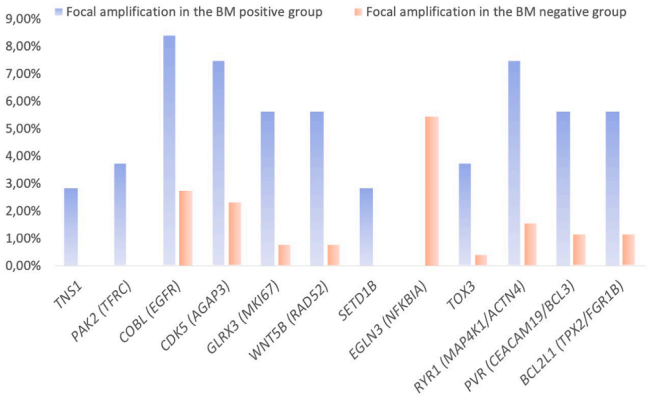

Fig. 1. Representation of focal amplifications. (a) Schematic representation of significant focal amplification variations between the brain metastasis (BM)-positive and BM-negative groups, with adjacent interest region in brackets. (b) Illustration of focal amplification in the *BCL2L1* gene.

3.4. Baseline NGS analysis

In both the BM-positive and BM-negative groups, a median of two molecular variants was identified in each sample (means 2.16 and 2.25, respectively). According to the predefined criteria (depth of coverage > 100X, allelic ratio > 5%, and population frequency < 0.1% in either 1000 g, ESP or gnomAD), 23 genes were selected for analysis: *ATM, BRAF, BRCA1, BRCA2, CTNNB1, EGFR, ERBB2, ERBB3, FBXW7, KEAP1, KRAS, MAP2K1, MAP2K4, MAP3K1, MET, NF1, NFE2L2, NRAS, PIK3CA, PIK3R1, STK11, TP53* and *VHL*. *KRAS* and *TP53* were the most frequently mutated genes in both the BM-positive (44.3% and 43.4%, respectively) and BM-negative groups (32.3% and 40.2%, respectively). Comparing the BM-positive and BM-negative populations, only *KRAS* mutations were found to be significantly associated with the presence of BMs ($p = 0.05$). *KRAS* mutations were found in 131 of the 360 patients

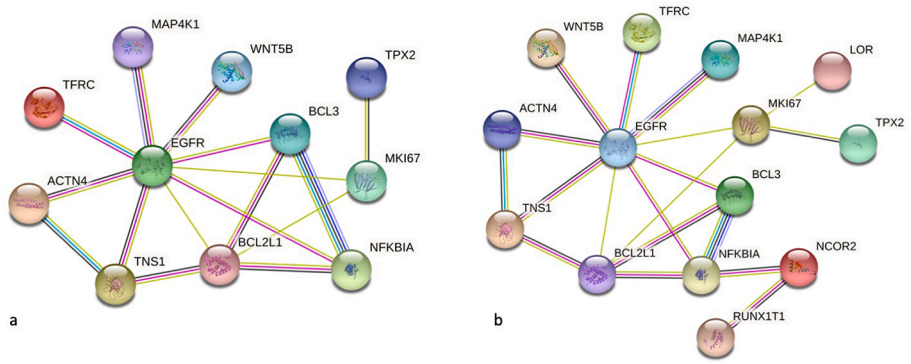

Fig. 2. Protein–protein interaction networks between the products of 11 of the 24 genes identified in the present study (a) and three of the 13 genes identified in our pilot study (b). Interactions as represented as follows: green line, literature co-occurrence; black line, co-expression; pink line, experimental interaction; blue line, public database association. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analyzed with NGS (36.4%), including 47 patients in the BM-positive group (44.3%) and 84 in the BM-negative group (33.1%). Distribution of *KRAS* mutations in both groups is summarized in Fig. 3. We observed a higher prevalence of the *KRAS* G12C mutation in the BM-positive group (63%). In the BM-negative group, although the *KRAS* G12C mutation remained dominant (47%), we observed a higher proportion of *KRAS* G12V (21%) and *KRAS* G12A (12%) mutations.

Over-representation of *KRAS* mutations among BM-positive patients was analyzed (on a sample of 361 patients because four patients lacked smoking status) in a series of multivariate analyses including smoking status (never/former versus current smokers), ECOG status (0 versus 1 or 2), sex (male versus female), age at diagnosis (continuous) and initial number of metastases (continuous). Adding an interaction term between BM status and smoking status, *KRAS* mutation was significantly associated with smoking status among those with BM ($p = 0.025$). In the same model, we found an association of *KRAS* status and sex and number of metastatic sites (OR = 0.60 [IC95% 0.38–0.95] $p = 0.031$ and OR = 1.29 [IC95% 1.01–1.64] $p = 0.043$, respectively). We also tested the interaction between BM status and other variables in other models, and the results did not show any significant interaction between these variables and *KRAS* mutation (data not shown). Finally, over-representation of KRAS mutations in the BM-positive group is certainly due to confounding factors.

3.5. CNS outcome during the maintenance phase

Out of the 365 patients included in our study, 153 (41.9%) were randomized, including 47 patients with baseline BMs and 106 without baseline BMs. Among them, 137 (89.5%) experienced disease progression before radiological data cutoff, planned after 18 weeks of maintenance therapy. Forty out of 47 patients (85.1%) with baseline BMs and 97 out of 106 (91.5%) without BMs experienced disease progression. Brain evolution data were available for 124 out of the 137 patients who experienced disease progression. Brain progression was observed for 17 patients in the BM-positive group (44.7%) and nine patients in the BM-negative group (10.5%). The occurrence of brain progression per treatment arm is summarized in Fig. 4a. Among the 89 randomized patients in the experimental arm (arm A of both substudies 1 and 2), 20 patients (22.5%) experienced brain progression, versus six out of 35 randomized patients (17.1%) in the standard chemotherapy arm (arm B of both substudies 1 and 2). Regardless of the initial BM status, the highest proportion of brain progression was identified in the durvalumab arm (substudy 2, Arm A), accounting for seven CNS progressions (28%) (Fig. 4b).

When we focused on the *KRAS* mutations, G12C mutation was found in eight patients among the 12 *KRAS*-mutant NSCLC patients experiencing brain progression, thus representing 66% of the mutations. On the other hand, G12C mutation accounted for 38% of *KRAS* mutations in the population without brain progression (G12V 26% – G13C 24%) regardless of the initial BM status.

4. Discussion

Given the high frequency, morbidity and mortality related to lung cancer BMs, there is a need for better characterization of BM molecular profiles and a better understanding of CNS outcomes in patients undergoing systemic treatments. We conducted the largest study to date assessing the molecular profiles of lung cancer patients with BMs. Comparative analysis of focal amplifications identified a 12-gene signature – enhanced by 12 adjacent genes (including *EGFR*) – that was statistically significantly associated with the presence of BMs in NSCLC. We designed the discovery strategy in such a way that every

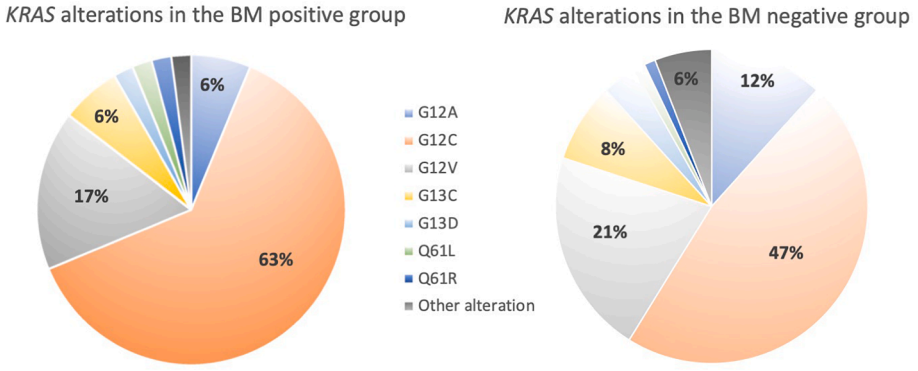

Fig. 3. Distribution of *KRAS* alterations based on brain metastasis (BM) status.

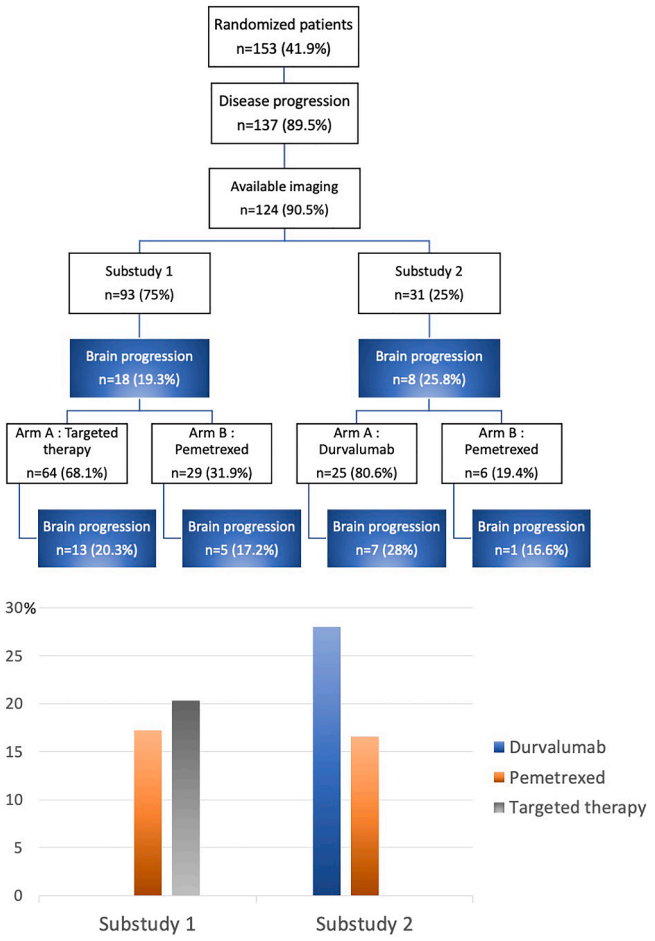

Fig. 4. CNS outcome during the treatment phase. (a) Flow chart of brain progression according to maintenance treatment type received. Substudy 1: presence of an actionable molecular alteration. ARM A: experimental treatment with targeted therapy; ARM B: standard treatment with pemetrexed. Substudy 2: absence of an actionable molecular alteration or contraindication to targeted therapy. ARM A: experimental treatment with durvalumab; ARM B: standard treatment with pemetrexed. (b) Histogram representation of brain progression according to maintenance treatment type received.

selected gene has a functional implication in the oncogenic process, thus strengthening the clear involvement of the signature in the brain dissemination process. Moreover, protein analyses highlighted several interactions not only between the identified genes but also with three genes of the brain-specific signature previously identified within our pilot study [11]. The main strengths of this work are the prospective and randomized design of the phase II SAFIR02-LUNG trial and the superior quality and quantity of molecular data. Furthermore, samples were extracted from primary lung tumors or various distant metastatic sites, representative of routine samples used for molecular testing.

All 24 genes identified using the discovery strategy and associated with the presence of baseline BMs have previously been described as a part of the oncogenic process and thus are possibly involved in brain metastatic invasion. These genes are implied in several main oncogenic pathways, corroborating the biological relevance of the signature. For example, *TPX2*, *TNS1* and *CEACAM19* are involved in the PI3K/AKT/mTOR pathway and have been described in various tumor types as being associated with aggressive disease [20–22]. *TFRC*, encoding the transferrin receptor TFR1, is a key regulator of the ferroptosis mechanism [23]. The specific role of TFR1 has been described in brain oncogenesis since the early stages and is associated with poorer outcomes [24,25]. *MKI67*, *TNS1, WNT5B* and *ACTN4* have been described in the proliferation process [26,27]. *RAD52* (homologous recombination), *CDK5* and *BCL2L1* are involved in the DNA damage repair process, thus impacting cancer outcome and treatment efficacy [28–31]. *PVR* is known as part of the TIGIT immune checkpoint axis, and *PVR* overexpression is associated with aggressive breast cancer subtypes [32]. *MAP4K1* is involved in the JNK/c-Jun pathway, regulates cell invasion and migration, and is associated with cisplatin resistance [33]. Moreover, some of the identified genes are also described in neurological processes. *CDK5* is known to be a key regulator of cytoskeletal remodeling, neuronal signaling and brain development, and is involved in several neurodegenerative processes [34]. *AGAP3* is a component of the NMDA receptor complex that regulates Ras/ERK signaling and is responsible for synapse strengthening [35]. Finally, *EGLN3,* which promotes HIF degradation, is involved in glioma vascular normalization and invasiveness [36], and *BCL3* overexpression is associated with a more malignant phenotype in glioblastoma and a lower response to temozolomide by inducing an epithelial–mesenchymal transition [37].

The biological relevance of our signature was enhanced by protein interaction analyses. Indeed, we observed a major role for focal *EGFR* amplification (8.4% versus 2.7%) as a brain dissemination driver, although activating mutations were excluded from the study. Analysis of *EGFR* focal amplification was sufficient to determine the association between *EGFR* amplification and the presence of baseline BMs. The interaction with several other proteins also related to the presence of BMs (WNT5B, MAP4K1, TFRC, ACTN4, TNS1, BCL3 and BCL2L1) and strengthens the implication of *EGFR* in the brain metastatic process. In the neuro-oncology field, *EGFR* amplification, found in>40% of glioblastomas, has been described for many years as an early progression driver [38,39]. Protein interactions other than EGFR mostly involve the DNA damage repair pathway or cellular proliferation, without clear functional co-occurrence in lung or brain malignancies in the literature to date. We also succeeded in highlighting protein interactions between our signature (*via* KI67 and NFKBIA) and products from genes previously identified as specifically altered in BM tissue [11]. Despite the lack of clear involvement of *LOR* in the oncogenic process, *NCOR2* was described as a transcription-repressive regulator in lung adenocarcinoma, and thus appears to be a tissue-dependent tumor regulator [40]. Unfortunately, there are no available data about *NCOR2* function in BMs or primary brain tumors. Moreover, *RUNX1T1* has been described to be involved in neuronal differentiation *in vitro*[41] and in epigenetic regulation in small-cell lung cancer [42], in addition to being involved in the liver metastatic process of pancreatic endocrine malignancies [43].

Comparative NGS analyses identified a higher proportion of patients harboring *KRAS* mutations in the BM-positive group. This result was unexpected, given the alleged protective role of *KRAS* mutations on brain dissemination [8]. This result may be explained by the higher proportion of active smokers in the BM-positive group, as confirmed by multivariate analysis, as well as the distribution of *KRAS* mutations in our study. Indeed, the fraction of G12C mutations was higher in the BM group (63% versus 47%) and even greater in the group of patients who had CNS progression (73% versus 38%). Growing evidence in *KRAS* biology highlighted an association between the G12C mutation and a lower level of AKT phosphorylation compared to other *KRAS* mutations, which is offset by activating the MEK pathway [44]. Despite the lack of a clear relationship between the MEK pathway and the brain dissemination process, this biological characteristic could be at least partially responsible for the high incidence of G12C mutation in the BM group. Moreover, the *KRAS* G12C mutation is already known to have a poorer prognosis and a decreased response rate to platinum-based chemotherapy than other *KRAS* mutations, possibly because of the implication of the MEK pathway [45,46]. Interestingly, inhibitors of *KRAS* G12C are now available in the clinic that demonstrated efficacy even in pretreated patients with BMs. [47].

CNS progression data also highlighted, as expected, a higher rate of CNS progression among patients with baseline BMs (44.7% versus 10.5%). Although the baseline prevalence of BMs in our study is

consistent with published data (29.3% in our study versus 26.8% in the review published by Cagney *et al*. [48]), we observed a lower rate of CNS progression after first-line treatment. This result can be explained by the exclusion of patients harboring symptomatic BMs at baseline or progressive BMs at randomization in the SAFIR02-LUNG trial. As a result, the small sample size of the brain progression subgroup did not allow us to perform statistical analyses to identify molecular features of CNS progression in each substudy arm. However, we observed a lower rate of brain progression in both substudies 1 and 2 among patients randomized in the pemetrexed arm (standard treatment). Superior pemetrexed CNS efficacy has already been described in several clinical trials compared with other chemotherapy regimens [49,50]. Although impressive brain efficacy was reported in patients undergoing targeted therapies against common types of molecular alterations, especially *ALK* and *EGFR* [51,52], CNS progression was slightly higher in our study in the targeted therapy arm. This can be explained by a lack of systemic efficacy of the study drugs, pharmacokinetics and pharmacodynamics of the therapeutics, or the brain prognosis associated with the specific alterations. The highest rate of CNS progression was observed in the durvalumab arm. Despite the systematic exclusion of patients harboring BMs from the main ICI clinical trials because of the frequent use of corticosteroids, data regarding the CNS efficacy suggested as in this work, previously reported by our team, that the BM control in patients treated with ICI was dependent on additional focal brain treatment (surgery, radiosurgery, stereotactic irradiation), which was optional for inclusion in the SAFIR02-LUNG trial [16]. CNS control with ICIs needs to be further explored, especially to identify predictive biomarkers of systemic and intracranial responses, considering the highly specialized immune environment in the brain [53]. For example, microglia ensure local innate immunity, and astrocytes represent essential adaptative CNS effectors. Indeed, preliminary data showed significant pembrolizumab brain efficacy even without focal brain treatment among NSCLC patients (brain response 33%) [54].

We identified a few limitations to our study. First, patient selection based on the CNS status may not be representative of BM patients encountered in daily care, who are often symptomatic at the time of diagnosis. The use of a limited NGS panel for molecular analysis may also be an issue. Full exome analysis is now within reach for clinical trials and may have provided valuable information. Finally, exploring the molecular profiles of BMs without paired BM tissue could be considered a scientific limitation. However, given the difficulties in obtaining brain tissue, the molecular profiles of BMs will have to be extrapolated from primary tumors to have a clinical impact.

Moving forward, transferring the outcome of this work to a routine unselected population of patients who have undergone large NGS analysis would be interesting as a validation cohort. Then, functional analysis of this preliminary signature would help us evaluate the implication of the identified genes in the brain metastatic process. First, we could identify potential therapeutic targets for lung cancer BMs to improve prognosis in the case of CNS dissemination and to prevent BMs in the adjuvant setting if a molecular profile of CNS susceptibility is identified in the primary tumor. Despite progress made in understanding the biology of NSCLC and BMs, active tobacco smoking remains a major risk for developing BMs that worsen NSCLC prognosis, confirming the importance of tobacco cessation policies in the prevention of NSCLC.

In conclusion, we conducted the largest study to date to assess the molecular features of lung cancer with BMs, and we successfully identified a 24-gene signature whose focal amplification is associated with the presence of baseline BMs and all involved in the oncogenic process. In the near future, the identification of a specific BM signature in primary tumors could help to prevent CNS progression and improve NSCLC outcomes.

CRediT authorship contribution statement

Alice Mogenet: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **Fabrice Barlesi:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Benjamin Besse:** Data curation, Investigation, Project administration, Resources, Writing – review & editing. **Stefan Michiels:** Conceptualization, Data curation, Formal analysis, Methodology, Resources, Software, Supervision, Validation, Writing – review & editing. **Maryam Karimi:** Data curation, Formal analysis, Methodology, Software, Writing – review & editing. **Alicia Tran-Dien:** Data curation, Formal analysis. **Nicolas Girard:** Data curation, Investigation, Writing – review & editing. **Julien Mazieres:** Data curation, Investigation, Writing – review & editing. **Clarisse Audigier-Valette:** Data curation, Investigation, Writing – review & editing. **Myriam Locatelli-Sanchez:** Data curation, Investigation, Writing – review & editing. **Maud Kamal:** Formal analysis, Methodology, Writing – review & editing. **Pierre Gestraud:** Formal analysis, Methodology, Writing – review & editing. **Abderaouf Hamza:** Formal analysis, Methodology, Writing – review & editing. **Alexandra Jacquet:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing. **Marta Jimenez:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing. **Sabrina Yara:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing. **Laurent Greillier:** Data curation, Investigation, Writing – review & editing. **François Bertucci:** Data curation, Investigation, Writing – review & editing. **David Planchard:** Data curation, Investigation, Writing – review & editing. **Jean-Charles Soria:** Data curation, Investigation, Writing – review & editing. **Ivan Bieche:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Pascale Tomasini:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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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Appendix A. Supplementary data

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