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Original Research

Mutational profiles of primary pulmonary adenocarcinoma and paired brain metastases disclose the importance of *KRAS* mutations☆

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KEYWORDS
Brain metastases;
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Abstract ***Background:*** Brain metastases present a significant complication in lung cancer with an unmet therapeutic need.
Methods: In this single-centre, retrospective study, we genotyped a clinico-pathologically well-annotated cohort of consecutively resected brain metastases of lung adenocarcinomas and

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Next generation sequencing;
KRAS G12C

paired primary tumours, diagnosed from 2000 to 2015, using the Ion Torrent Oncomine Comprehensive Cancer Panel v3.

Results: Among 444 consecutive brain metastases, 210 (49%) originated from lung cancer. Analysis was successful in 111 samples, including 54 pairs of brain metastasis and primary tumour. Most driver alterations were preserved in brain metastases. Private alterations exclusive to primary tumours, brain metastases or both sites (intersecting cases) were present in 22%, 26% and 26% of cases, respectively. Seven percent had no shared mutations. KRAS mutations were more frequent in primary tumours metastasised to the brain (32/55, 58%) compared to TCGA (33%, $p < 0.005$) and own data from routine diagnostics, independent from clinical or pathological characteristics. Fourteen cases showed alterations in the EGFR signalling pathway including additional KRAS alterations that were private to brain metastases. *KRAS* G12C was detected most frequently (26% of patients) and *KRAS* G12C and G13C variants were significantly enriched in brain metastases. Synchronous and metachronous cases had a similar mutation profile.

Conclusions: Our results suggest an important role of *KRAS* alterations in the pathobiology of brain metastases from lung adenocarcinomas. This has direct therapeutic implications as inhibitors selectively targeting KRAS G12C are entering the clinics.

1. Introduction

Lung cancer is the most frequent and deadly malignant disease worldwide, and the most frequent origin of brain metastases [1]. Up to 40% of patients with non-small cell lung cancer (NSCLC) will develop brain metastases in the course of their disease [2]. Lung adenocarcinomas (LUAD) and neuroendocrine lung carcinomas are the subtypes most prone to spread to the brain [3,4].

The current therapeutic options are limited and curative strategies are restricted to patients with limited intra- and extracranial disease, resting upon (radio-) surgery. In patients with multiple symptomatic brain metastases, due to low benefit from chemotherapy, whole brain radiotherapy (WBRT) remains the standard of care, despite limited evidence from randomised trials and risk for neurocognitive decline [5]. In advanced NSCLC, immune checkpoint inhibitors are an effective alternative or addendum to chemotherapy, but only limited data is available for patients with brain metastases [5]. Oncogene addicted NSCLC are especially prone to metastasise to the brain and in patients with druggable oncogenes, next-generation tyrosine kinase inhibitors are a valuable choice inducing potent and durable responses [6].

Hence, therapeutic strategies for most patients with NSCLC with dissemination to the brain are unsatisfactory and 5-year survival remains poor [7]. One major obstacle to overcome is our lack of in-depth knowledge of the molecular mechanisms implied in the development of brain metastatic disease. Despite multiple in vitro and animal-based studies, molecular studies on human tissue are scarce and only recently emerging [8–11]. Brastianos and colleagues demonstrated a branched evolution of brain metastases, highlighting the importance of genomic characterisation of both the primary tumour and the metastasis [12]. A recent study on 32 matched pairs of synchronous, untreated LUAD with brain metastases identified amplifications of *YAP1*, *MMP13* and *MYC* as potential brain metastatic drivers [13].

The unmet clinical need of better therapeutic and preventive strategies and the paucity of tissue-based studies inspired this study with the aim to uncover molecular mechanisms important for lung cancer brain metastases by molecularly characterising a well-curated cohort of paired LUAD brain metastases and primary lung tumours.

2. Materials and methods

2.1. Patient cohort

We analysed consecutive patients with resected lung carcinoma brain metastases, diagnosed at the Institute of Pathology, University of Bern from 2000 to 2015. All tumours were resected at the Department of Neurosurgery, Inselspital University Hospital Bern. The cohort was assembled according to the pathology reports and clinical files. Histology was reevaluated in all cases according to current guidelines [14]. Only tumours with adenocarcinoma diagnosis in the brain metastasis and sufficient material for molecular analyses from both the metastasis and the primary lung tumour were included in the cohort for molecular analysis. This comprised 57 patients: 55 with LUAD and 2 with adenosquamous carcinoma in the lung resections. All tumours were re-staged according to the UICC 8th edition of the TNM-classification [15]. Brain metastases were synchronous in 26 (46%) patients and metachronous in 31 (54%) patients, the latter defined as having occurred

at least 3 months after initial diagnosis [16]. At diagnosis, 34 patients were active smokers, 18 former smokers and 4 never-smokers. Detailed tumour characteristics of the cohort are provided in Table 1. Overall survival (OS) was defined as the period from initial diagnosis until death from any cause. Median OS was 36 months (95% CI 28–54 months; 46 events). The study was conducted and is reported according to the REMARK-criteria [17], and was approved by the Cantonal Ethics Commission of the Canton of Bern (KEK-BE: 2016-01497), which waived the requirement for written informed consent.

2.2. Next generation sequencing

Genomic DNA and RNA were extracted from tissue punches using the QIAamp DNA Microkit (Qiagen, Hilden, Germany) and the Ambion RevoverAll Kit (ThermoFisher Scientific, Waltham, Massachusetts), respectively. Next generation sequencing was conducted using the Oncomine Comprehensive Panel v3 (ThermoFisher Scientific). Libraries were constructed following the manufacturer's recommendations. Emulsion PCR and chip loading on Ion Chef 540 Chips was performed using the Ion Chef System (ThermoFisher

Table 1
Baseline characteristics of the study cohort according to KRAS mutational status in the primary tumour.

	KRAS WT (n = 23)	KRAS mutation (n = 32)	p-Value
Age, years (median [IQR])	**64 [53.5–68.5]**	**59.5 [51.8–64.2]**	**0.326**[a]
Sex	**n = 23 (%)**	**n = 32 (%)**	**1**[b]
Female	13 (57)	19 (59.4)	
Male	10 (43)	13 (40.6)	
Latency of BM	**n = 23 (%)**	**n = 32 (%)**	**0.783**[b]
Synchronous	11 (48)	13 (41)	
Metachronous	12 (52)	19 (59)	
Smoking status, pack years (median [IQR])	**45 [35–70]**	**40 [30–50]**	**0.623**[a]
Therapy between resection of primary tumour and BM	**n = 23 (%)**	**n = 32 (%)**	**0.272**[b]
Yes	8 (35)	17 (53)	
No	15 (65)	15 (47)	
pT-Descriptor	**n = 21 (%)**	**n = 32 (%)**	**0.372**[a]
pT1	3 (14)	9 (28)	
pT2	11 (52)	12 (38)	
pT3	1 (5)	7 (22)	
pT4	6 (29)	4 (12)	
pN-Descriptor	**n = 19 (%)**	**n = 32 (%)**	**0.804**[a]
pN0	5 (26)	9 (28)	
pN1	7 (37)	13 (41)	
pN2	7 (37)	9 (28)	
pN3	0 (0)	1 (3)	
TNM stage (at lung cancer diagnosis)	**n = 22 (%)**	**n = 32 (%)**	**0.057**[a]
I	1 (5)	1 (3)	
II	2 (9)	10 (31)	
III	5 (23)	7 (22)	
IVA	9 (41)	12 (38)	
IVB	5 (23)	2 (6)	
Location of BM	**n = 23 (%)**	**n = 32 (%)**	**0.907**[b]
Frontal	6 (26)	10 (31)	
Frontoparietal	1 (4)	0 (0)	
Temporal	2 (9)	3 (9)	
Temporooccipital	0 (0)	3 (9)	
Parietal	3 (13)	3 (9)	
Occipital	1 (4)	2 (6)	
Cerebellum	4 (17)	5 (16)	
Multiple unilateral	1 (4)	2 (6)	
Multiple bilateral	3 (13)	3 (9)	
Other	2 (9)	1 (3)	
Number of BM	**n = 23 (%)**	**n = 32 (%)**	**0.756**[b]
Single	17	25	
Multiple	6	7	
Radiological BM size, mm (median [IQR])	**31 [28–40]**	**28 [20.8–36.8]**	**0.219**[a]

Other locations include meninges and hypophysis. IQR, Interquartile range; BM, Brain metastasis.
[a] Mann–Whitney-U test.
[b] Fisher's exact test.

Scientific). Sequencing was performed using the Ion S5 Sequencing Kit and the Ion S5 Instrument (ThermoFisher Scientific).

Sequencing reads were aligned with the human genome reference (hg19) using the Torrent Suite software v5.6 (ThermoFisher Scientific). Minimal target base coverage at 100× was set to 95%. Variant allele frequency threshold was set to 5%. Variant annotation was performed using the IonReporter Oncomine Comprehensive v3 v5.6 Workflow (ThermoFisher Scientific). Minor allele frequency threshold was set to 10^{-4}. Pathogenicity of alterations was assessed using Varsome (https://varsome.com/) and cBioPortal (https://www.cbioportal.org/).

2.3. Pathway analysis

Gene set enrichment analysis was performed following the fgsea pipeline (min size = 5, max size = 20, permutations = 10,000). Input pathways included consensus.gmt files for KEGG, and GO terms as previously described [18]. No custom code or mathematical algorithm that is deemed central to the conclusions was used.

2.4. Statistical analysis

R software (version 4.0.4, https://cran.r-project.org) was used for statistical analysis. We performed Fisher's exact tests for group comparisons. Mann–Whitney-U and Kruskal–Wallis test were applied for comparison of naturally ordered and numeric variables. Survival analysis was done using Kaplan–Meier estimates and log rank test. Two-sided p-values of <0.05 were regarded as significant.

3. Results

3.1. High frequency of KRAS mutations in LUAD metastatic to the brain

Next generation sequencing was successful in 54 tumour pairs and failed in 2 primary tumours and one brain metastasis. We identified 216 missense mutations, 58 truncations, 7 inframe indels, 72 copy number variations, and 5 fusions (Supplementary Table S1). The most frequent alterations in primary tumours were missense mutations and truncating mutations in *TP53* (33/55, 60%), activating *KRAS* mutations and amplifications (32/55, 58%), *MYC* mutations and amplifications (9/55, 16%) and *STK11* missense and truncating mutations (9/55, 16%) (Fig. 1A). The frequency of *KRAS* mutations detected at primary sites was significantly higher compared with TCGA (33%) and with own data derived from routine clinical diagnostics, where we detected 41 cases (35%) in all 116 evaluated UICC-stage IV lung cancer samples analysed in 2017, and 33/95 (35%) cases when excluding systemic metastases. The TCGA-dataset comprises sequence data from LUAD without brain metastases [19]. The proportions of former or present smokers in the TCGA dataset (81%) and our cohort (91%) were similar. Out of seven lung tumours resected following neoadjuvant chemotherapy, only three were *KRAS*-mutated. This excludes smoking history and chemotherapy as possible explanations for the high frequency of *KRAS* mutations.

It was only the frequency of *KRAS* mutations that differed statistically significantly between our cohort and the TCGA cohort ($p < 0.005$), and the *KRAS* G13C variant was the only one more prevalent than expected from TCGA ($p < 0.02$; Fig. 2). All other alterations were in the range expected, except from *EGFR* mutations, which were less frequent (4/55, 7%; TCGA: 16%).

3.2. KRAS among the most frequent secondary alterations exclusive to brain metastases

Frequencies of *TP53*, *KRAS*, *MYC* and *STK11* alterations as compared to TCGA were even higher in brain metastases than in primary tumours (Supplementary Fig. 1). *KRAS* mutations and *MYC* copy number gains at the metastatic site were significantly more frequent and *RICTOR* copy number gains were significantly less frequent than expected from TCGA ($p < 0.05$; Fig. 2). Among activating *KRAS* mutations, *KRAS* G12C ($p < 0.05$) and G13C variants ($p < 0.005$) were significantly enriched. The prevalence of *KRAS* G12C mutations or other *KRAS* alterations in primary tumours was not associated with smoking history as measured by pack years (Supplementary Fig. 2A), nor were *MYC* or *RICTOR* amplifications (Supplementary Fig. 2B and C).

We assessed whether *KRAS* mutations were associated with latency for the manifestation of brain metastases relative to the primary tumour. A trend towards longer latency was noted for patients with *KRAS* alterations ($p = 0.084$), but no difference was observed between *KRAS* G12C, *KRAS* G13C or other *KRAS* alterations (Supplementary Fig. 3A). Likewise, no difference in the incidence of *MYC* or *RICTOR* amplification was observed for synchronous or metachronous cases (Supplementary Fig. 3B and C). Presence of *KRAS* alterations in the primary tumour had no impact on OS (Supplementary Fig. 4).

3.3. Clonal evolution of brain metastases

The comprehensive cancer v3 panel covers most cancer genes relevant for LUAD. Based on the occurrence of shared and private mutations, copy number variations and gene fusions between the primary site and the brain metastasis, we defined five different molecular subgroups of LUAD (Fig. 3). All molecular subgroups are driven by *KRAS* and *TP53* alterations. Ten/54 (19%)

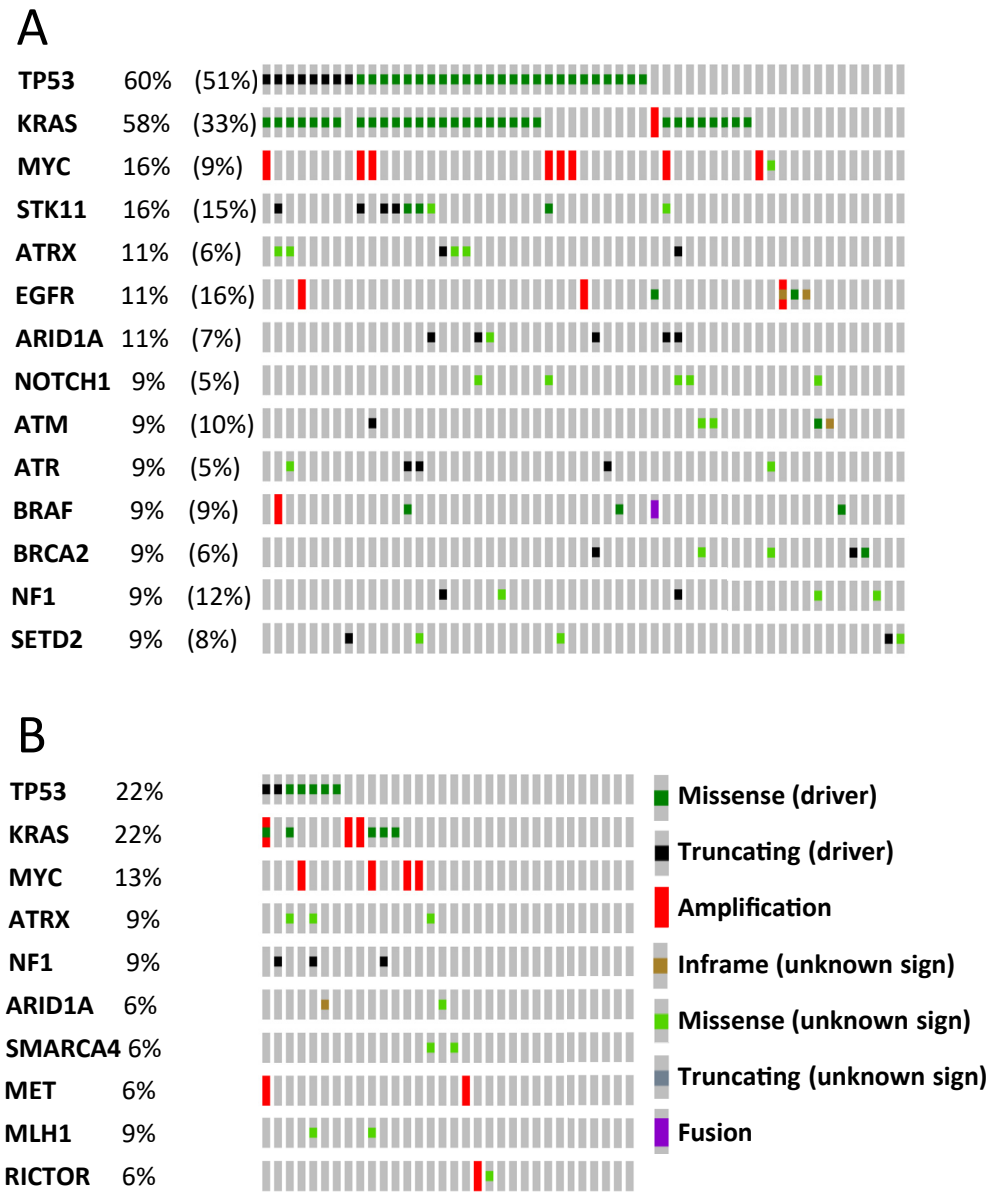

Fig. 1. Oncoprint of genetic alterations in the primary lung carcinoma site and those private to brain metastases. (A) Genetic alterations in the lung resections from LUAD with brain metastases show a much higher frequency of *KRAS* alterations than expected from the TCGA dataset (numbers in brackets). (B) Genetic alterations exclusive to the brain metastases from LUAD as compared to paired primary lung tumours. Note that components of EGFR signalling pathways including *KRAS*, *MYC*, *NF1*, *PIK3CA* and *RICTOR* are among the most frequently mutated genes.

cases showed the same set of mutations in the primary and metastatic sites with no evidence for private mutations (P = BM). *TP53*, *KRAS* and *MYC* were the most frequent alterations in this group. Twelve/54 (22%) cases showed private alterations exclusively in the primary tumour, with *RICTOR* being the most frequently occurring private alteration (P > BM). Fourteen/54 (26%) cases showed private alterations exclusively in the metastatic site (P < BM), but no enrichment for genes affected by private mutations. Intersecting cases (14/54, 26%) are defined by private mutations at both primary and metastatic sites (P∩BM). Interestingly, here private mutations at the metastatic site frequently harboured additional *KRAS* mutations (21%) and *KRAS* copy number gains (21%). In contrast, private *KRAS* alterations in the primary tumour were observed only in one case (1/54). Finally, the P/BM category (4/54, 7%) consisted of pure LUAD tumour pairs with no shared mutations. This group may consist of collision tumours. All P/BM tumour pairs harboured the same sets of genetic polymorphisms, confirming their origin from the same patient.

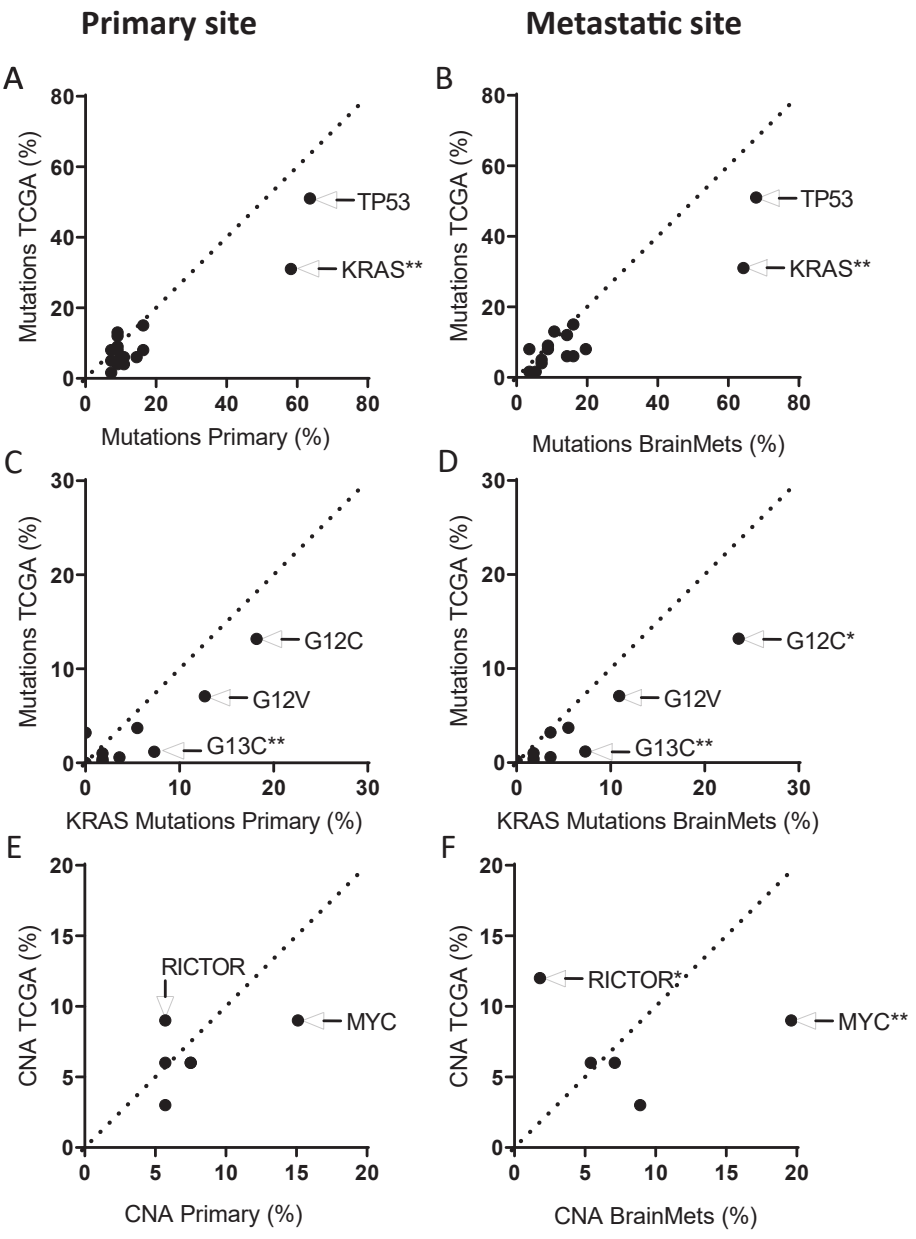

Fig. 2. Frequencies of genetic aberrations in the primary site and metastatic site of lung adenocarcinoma cases with brain metastases in comparison with the TCGA dataset. (A–B) Analyses of mutations at the primary site and in the brain metastases show the significantly enhanced frequency of *KRAS* mutations as compared to TCGA. (C–D) Specification of the KRAS mutations discloses a significantly higher frequency of the *KRAS* G13C mutations in both locations and *KRAS* G12C in the brain metastases. (E–F) Analysis of copy number alterations (CNA) shows significantly more frequent *MYC* amplifications in the brain metastases. Genes with alteration frequencies >8% are indicated. *, $p < 0.05$; **, $p < 0.005$.

There was no difference in latency of manifestation of brain metastases between the molecular subgroups (Supplementary Fig. 6).

3.4. Private alterations of the EGFR pathway are confined to the metastatic site

Among tumours with private mutations at the metastatic site (P < BM and P∩BM tumours), 14/28 (50%) tumours harboured one or several alterations in genes involved in the EGFR signalling pathway that were confined to the metastatic site. These alterations included activating mutations (n = 3) and CNA (n = 3) of the *KRAS* gene, activating mutation or CNA in *PIK3CA* (n = 2), CNAs in *MET* (n = 2), *RICTOR* (n = 2) and *AKT2* (n = 1), *EGFR* Exon 2–7 skipping variant EGFRvIII (n = 1) and *NF1* truncating mutations (n = 2) (Supplementary Table S1). Fig. 4 shows

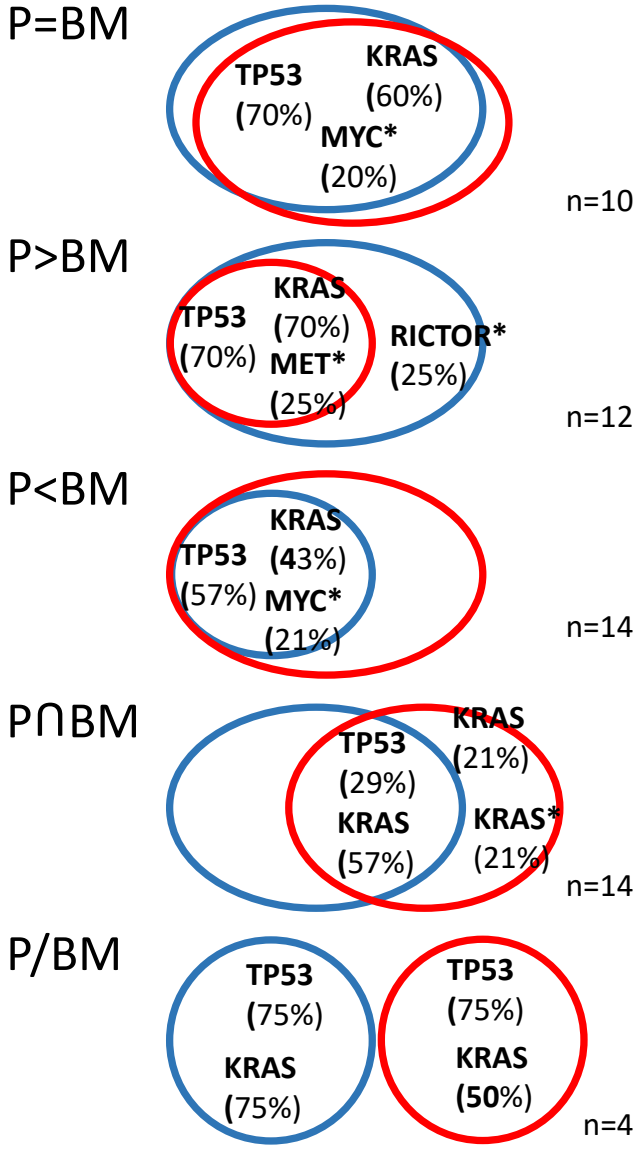

Fig. 3. Unique and shared mutations and copy number alterations between primary site (P, blue line) and brain metastases (BM, red line) of lung adenocarcinoma. P = BM, patients with same mutations at the primary and metastatic site; P > BM, private mutations at the primary tumour site; P < BM, private mutations in the brain metastasis, P∩BM, intersecting cases with private mutations at both sites, P/BM, no shared mutations. Only the most frequent alterations are indicated. *, CNA.

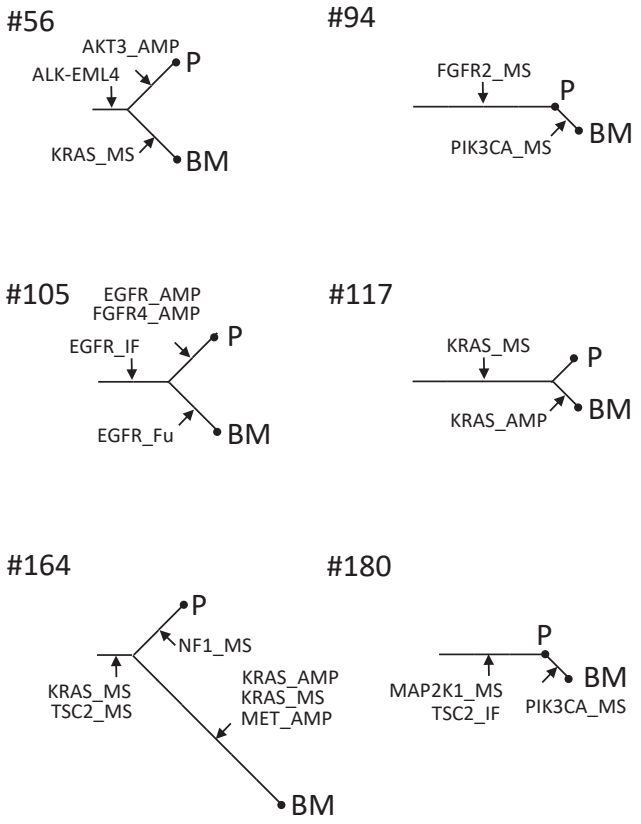

Fig. 4. Phylogenetic trees of primary site (P) and brain metastasis (BM) of lung adenocarcinoma cases with brain metastases. Genes of the EGFR signalling pathway with missense mutations (MS), in frame indels (IF), truncations (TR), amplifications (AMP) and gene fusions (FU) are indicated in the trunk (horizontal line) and branches (angled lines). Tumours #56, #105 and #164 contain private alterations in both locations while tumours #94, #117 and #180 contain private alterations exclusive to the brain metastases. Note that private and shared alterations in the EGFR signalling pathway can co-occur.

examples of tumours with private mutations of components of the EGFR signalling pathway.

3.5. Pathway analysis of mutational profile

We finally assessed the mutational profile of primary tumours and brain metastases for their implication in cellular processes. Gene set enrichment and gene ontology analysis of mutations identified in primary tumours and metastases but not enriched in the TCGA dataset revealed a most significant enrichment for colorectal cancer genes (consisting mainly of genes involved in EGFR signalling pathway), IL2 pathway, LKB1 pathway, cytokine-mediated signalling pathway and response to cytokines (Supplementary Figs. 7 and 8).

4. Discussion

We report a comprehensive genetic analysis of matched LUAD and brain metastases. *KRAS* mutations and copy number gains were significantly increased in primary tumours metastasised to the brain (58%) compared to our in-house control cohort of UICC stage IV tumours (35%), the TCGA dataset and other reported stage IV cohorts (21–40%) [20–22]. This is in line with previous studies. NGS analysis of 72 isolated LUAD brain metastases identified *KRAS* mutations among the most commonly mutated genes [23]. Contrary, no enrichment for *KRAS* alterations was detected in 54 stage IV LUAD primary tumour–metastases

pairs, including only 7 brain metastases [11]. The increased frequency of *KRAS* mutations is not unique to LUAD with brain metastases. It was also reported in colorectal cancer with brain metastases compared to stage IV disease without brain involvement [24]. Among the *KRAS* mutations we identified, G12C was the most frequently detected variant (n = 14/39, 36%), present in 26% of the entire cohort. In contrast, the prevalence of the G12C variant in LUAD with metastases to other sites is estimated to be around 13% [25–27]. G12C is associated with smoking, in line with heavy smokers being more frequently affected by brain metastases than nonsmokers [28,29]. It was only recently that specific oral KRAS G12C inhibitors were developed, resulting in several promising drugs currently in advanced clinical trials [30–33]. This increases the clinical significance of our findings.

One major advantage of our study compared to literature is the availability of paired samples from primary tumours and brain metastases in a clinico-pathologically well-annotated cohort. We provide evidence for a high incidence of mutations private to brain metastases, with a bias towards the EGFR signalling pathway. Notably, tumour #56 presented with a private *KRAS* mutation in the synchronous and untreated brain metastasis in addition to the *ALK-EML4* translocation common to both the primary and metastatic site. Likewise, tumour #164 harboured two independent *KRAS* mutations, one common to both sites and the other private to the brain metastasis. These findings are in marked contrast to LUADs metastatic to other locations, for which occurrences of such alterations are mutually exclusive. Co-occurrence of *STK11* (LKB1) and *KRAS* mutations were reported to enhance EGFR signalling pathways [34], but LUAD metastatic to the brain have not been assessed in this regard. Applying pathway analysis, we showed the LKB1 pathway to be significantly enhanced in our cohort, suggesting a role in LUAD brain metastases. Interestingly, one patient with *HER2* amplified breast cancer was reported having acquired a private *EGFR* L858R mutation while a second patient with serous ovarian cancer acquired private *HER2*, *BRAF* and *FGFR1* amplifications exclusively in the brain metastases [12], suggesting that different alterations in the EGFR signalling pathway specific for brain metastases may present a general mechanism in solid cancer.

Enhanced EGFR signalling is a hallmark of cancer associated with invasion and metastasis [35], but the reason for brain tropism is unclear. Metastasis map analysis of human cancer cell lines for organ-specific patterns of metastasis revealed evidence for altered lipid metabolism in breast cancer cell lines capable of metastasising to the brain, a finding that also applies for primary brain tumours [10]. Interestingly, oncogenic KRAS activates fatty acid synthase in LUAD [36], but it remains to be shown if this is directly linked to developing brain metastases.

Additionally, *MYC* is a functionally validated driver of metastatic LUAD, and its amplification has been associated with brain metastases [13]. We confirm the enhanced incidence of *MYC* amplification in our cohort of LUAD metastatic to the brain.

Mutational analysis of 136 patients with systemic metastases from breast, colorectal and lung cancer revealed that metachronous metastases are associated with a higher number of private mutations at the metastatic site compared to synchronous cases, but brain metastases were not specifically addressed [37]. Based on the mutational profile, we subdivided our patient cohort into four major subgroups: patients with same sets of mutations at the primary and metastatic site (P = BM), private mutations exclusive to the primary and metastatic sites and intersecting cases with private mutations at both sites. Surprisingly, synchronous cases were not associated with the P = BM group but were comprised of all patient groups confirming the heterogeneous nature of LUAD. Four patients showed distinct mutations in the primary site and brain metastasis, a finding previously described by others [38].

In summary, our results suggest a major role of KRAS and other components of the EGFR signalling pathway in the pathobiology of brain metastases of LUAD. Our finding of *KRAS* G12C being the most frequent alteration might have direct clinical implications due to recently introduced effective inhibitors targeting KRAS G12C with sufficient penetration across the blood–brain barrier [39].

Data availability

Detailed clinico-pathological data and R-scripts for the data analysis are available upon request to the authors.

Author statement

Erik Vassella: Conceptualisation, Methodology, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualisation, Supervision, Project administration; **Elham Kashani**: Software, Formal analysis, Writing – review & editing, Visualisation; **Philipp Zens**: Software, Formal analysis, Data curation, Visualisation, Funding acquisition, Writing – review & editing; **Alexandra Kündig**: Investigation, Data curation; **Christian Fung**: Investigation, Resources, Writing – review & editing; **Amina Scherz**: Investigation, Resources, Writing – review & editing; **Evelyn Herrmann**: Resources, Writing – review & editing; **Ekin Ermis**: Investigation, Writing – review & editing; **Ralph A. Schmid**: Resources, Writing – Review & Editing; **Sabina Berezowska**: Conceptualisation, Methodology, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualisation, Supervision, Project administration, Funding acquisition.

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Conflict of interest statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: SB has served as compensated consultant for Basilea, Eli Lilly, MSD and Roche (payment to institution) and has received research funding from Roche outside of the current project. Other authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2021.10.006.

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