Characterization of genetic alterations in brain metastases from non-small cell lung cancer

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Brain metastasis (BM) is the primary contributor to mortality in non-small cell lung cancer (NSCLC) patients. Although the findings of NSCLC genetic sequencing studies suggest the potential for personalizing therapeutic approaches, the genetic profiles and underlying mechanisms of BM progression remain poorly understood. Here, we investigated the genetic profiles of brain metastases from NSCLC in six patients with primary tumors and corresponding BM samples via whole exome sequencing and targeted panel sequencing. We have demonstrated considerable genetic heterogeneity between primary lung cancer and corresponding brain metastases specimens. High-frequency mutations were found in NOTCH2, NOTCH2NL, FANCD2, EGFR, and TP53. Additionally, EGFR and TP53 consistently exhibited high frequencies of mutation between primary tumors and corresponding brain metastases. The implication is that most of the genetic alterations may be acquired or lost during malignant progression, and the stable EGFR and TP53 mutational status between paired primary tumors and metastatic sites confirms that most mutations detected on analysis of the primary tumor or metastases are sufficient for clinical decision-making, and suggest there is no need to re-biopsy recurrent tumors or metastases for most NSCLC patients.

Lung cancer has become the most frequently occurring tumor and the leading cause of cancer-related death [1]. Eighty-five per cent of lung cancers are classified as non-small cell lung cancer (NSCLC). Adenocarcinoma cases account for the largest number of NSCLC patients [2]. Median survival for patients with NSCLC is 13 months [3], and metastasis is the critical contributor to mortality rate. The brain is one of the most common organs for lung cancer metastasis, in which metastasis indicates poor prognosis. Approximately 10–25% of patients with lung cancer have brain metastases when the cancer is first diagnosed, and 40–50% of these eventually develop brain metastases during the course of their disease [4]. The median survival of these patients decreases to 2 months [5].

Precision medicine and targeted therapies are defined as individual treatments based on an individual patient’s mutant molecular profiles. Genetic sequencing studies of NSCLC have demonstrated complex subclones with genetic heterogeneity, both within primary tumors and between primary and lymphatic metastases [6,7]. Nevertheless, little is known regarding genetic heterogeneity of brain metastases in NSCLC. Furthermore, the heterogeneity of molecular profiles between primary tumors and brain metastases or heterogeneity that develops during the malignant process may limit the efficacy of individual treatments.

Abbreviations
BM, brain metastasis; BP, biological process; CC, cellular components; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular functions; NSCLC, non-small cell lung cancer; SNV, somatic nucleotide variant; WES, whole exome sequencing.
Therefore, there is an urgent need to explore mutations in brain metastases and to determine individualized therapy regimens. This study focused on investigating the altered genes in brain metastases from NSCLC and explored potential targeted treatments for driver mutations.

We carried out whole exome sequencing (WES) and deep target panel sequencing on six primary tumors and their corresponding brain metastasis samples, respectively. Then, we systematically analyzed somatic nucleotide variants (SNVs) and predicted the possibly or probably pathogenic genes. We then performed bioinformatic analysis to explore potential brain metastasis (BM)-associated driver genes or SNVs, as well as enriched biological processes (BP), molecular functions (MF) and signaling pathways.

**Materials and methods**

**Clinical and sample characteristics**

All protocols of the study were approved by the International Review Board of Fudan University Affiliated Hua-shan Hospital. Only BM from NSCLC was included in this study. We included six patients with a mean age of 53.5 years at diagnosis (range, 31–64 years) who underwent surgery or biopsy and had pathologically confirmed NSCL carcinoma and corresponding brain metastases. For three patients, brain metastases developed 14–24 months after primary tumor surgery. All primary and paired-metastases tumor tissue specimens were subsequently investigated by next generation sequence whole exome analysis. Another three patients were diagnosed with lung cancer and brain metastases simultaneously. The experiments were undertaken with the understanding and written consent of each subject. The study methodologies conformed to the standards set by the Declaration of Helsinki. The details of clinical characteristics are displayed in Table 1.

**Table 1.** Clinical and histopathological characteristics. Interval: time between primary tumor and brain metastasis; tumor size: maximum diameter of the tumor; stage: classification of lymph nodes and distal organs metastasis status according to the international system for staging lung cancer; TNM: stage of initial diagnosis. CT, chemotherapy; ND, not determined; TNM, the first tumor, lymph node, metastasis classification.

| ID | P1 | P2 | P3 | P4 | P5 | P6 |
|----|----|----|----|----|----|----|
| Age (years) | 58 | 33 | 57 | 56 | 67 | 64 |
| Sex | Male | Male | Male | Female | Male | Male |
| Histological type | Adenocarcinoma | Adenocarcinoma | Adenocarcinoma | Adenocarcinoma | Adenocarcinoma | Adenocarcinoma |
| Interval | 16 months | 14 months | 24 months | Synchronous | Synchronous | Synchronous |
| Tumor size (cm) | | | | | | |
| Primary | 4.4 | 2.5 | 5.5 | 4.0 | 3.9 | 7.0 |
| Metastases | 2.8 | 5.0 | ND | ND | ND | ND |
| Smoker | No | No | No | No | No | No |
| Stage | IIA | IIIA | IIB | IV | IV | IV |
| TNM | T2aN0M0 | T2aN2M0 | T2bN1M0 | T2aN0M1b | T2aN0M1b | T3N0M1b |
| Treatment | Surgery + CT | Surgery + CT | Surgery + CT | Surgery + CT | CT | Surgery + CT |
| Primary Metastases | Surgery | Surgery | Surgery | Surgery | Surgery | Surgery |

**DNA extraction and exome sequencing**

Genomic DNA was extracted from formalin-fixed, paraffin embedded (FFPE) tissue sections using the QiAamp DNA FFPE Tissue Kit (QIAGEN, Frankfurt, Germany), according to the manufacturer’s protocol. DNA was assessed for quality and was qualified using NanoDrop and agarose gel electrophoresis. DNA libraries were created using the SureSelect XT Library Prep Kit (Agilent, Palo Alto, CA, USA), according to the manufacturer's protocol. Exome capture was performed with the Agilent SureSelect kit. The DNA library and exomes were sequenced on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). The capture and coverage of raw reads were mapped to a human genome reference provided by the Burrows-Wheeler Aligner and reference sequence version HG19 alignment. Indels were called using GATK, and all variants were annotated by ANNOVAR.

**Functional and pathway enrichment analysis**

SIFT, LRT and POLYPHEN (polymorphism phenotyping) were used to predict the impact of pathogenic somatic mutations on gene function. The DAVID v6.8 (Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov/) was used to analyze the shared pathogenic genes of corresponding primary tumors and metastases, including gene ontology (GO) BP, cellular components (CC) and MF. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was used to examine molecular interactions, reaction and relation networks.
of the shared pathogenic genes identified from primary tumors and paired metastases. Fisher’s exact test was performed; \( P < 0.05 \) was regarded as indicating statistical significance.

**Results**

**Genetic profiling of primary tumors and brain metastases by whole exome sequencing**

Exons account for 1% of the human genome but contain approximately 85% of disease-causing mutations. To investigate the driver alteration genes in brain metastases from NSCLC and potential targeted treatments of driver mutations, we compared 12 samples (primary tumor and matched brain metastases) by exome sequencing. Of these, six were identified by WES, covering 230 418 amplicons of 23 129 genes. Six others were identified by deep targeted next-generation sequencing of 390 cancer associated genes. WES is a gene analysis method that captures and enriches the entire exonic regions using sequence capture technology and performs high-throughput sequencing. WES has the advantages of high throughput and low cost, and so we analyzed the whole driver mutant exome profiles in three pairs of specimens using WES.

We carried out WES with an average depth of 375× (range: 323–399×) and identified an average number of 2055 exonic somatic variant sites and 1008 mutant genes in three pairs of primary tumors and corresponding brain metastases. In order to further understand the possibly or probably pathogenic mutations, we used three filtering steps to exclude the volume of non-pathogenic variants and all variations showing poor quality as follows: (a) read depth < 30, (b) synonymous mutation, and (c) variant frequency < 5%. For P1 primary tumor and matched brain metastasis samples, we identified 2104 (in 1045 genes) and 2052 (in 999 genes) exonic somatic variant sites, respectively, after filtration (Table S1). For P2 primary lung cancer and matched brain metastasis tissues, we identified 1900 (in 949 genes) and 2146 (in 1059 genes) exonic somatic variant sites, respectively, after filtration (Table S2). For P3 primary tumor and matched brain metastasis samples, we identified 2083 (in 1029 genes) and 2044 (in 1017 genes) exonic somatic variant sites, respectively, after filtration (Table S3). Of these, approximately 84.6% were non-synchronous SNVs, significantly more than the proportion that were deletions (7.4% on average), insertions (6% on average), stop-gain (1.9% on average), and stop-loss (less than 0.1% on average) alterations. The transitions A→G, T→C, G→A and C→T were significantly more common than transversions and other types of transitions, but there was a slight difference between primary and paired-brain metastasis mutations. In this WES cohort, the transition/transversion ratio was 1.73 on average (Fig. 1).

**Genetic profiling of primary tumors and brain metastases by targeted next-generation sequencing**

Compared to WES, a targeted sequencing panel only captures and enriches exonic regions of the targeted genes, but with better detection depth and accuracy. To increase the sensitivity of detection for known targeted gene abnormalities and to decrease the interference of treatments, a targeted sequencing panel was performed in another three untreated patients to identify mutations in genes that may be critical for brain parenchyma metastatic lung cancer. We then performed a deep targeted sequencing panel of 390 cancer-related genes at an average depth of 678× (range: 370–821×), including 80 targeted treatment genes, 200 genes involved in druggable target-related signaling pathways, 50 genes involved in DNA damage repair, 25 genes involved in epigenetics, 25 fusion genes, 10 other cancer-related genes (e.g. EGFR, TP53, Braf, PTEN, KRAS, and BRCA1). For P4 primary tumor and matched brain metastasis samples, we identified 64 (in 34 genes) and 70 (in 48 genes) exonic somatic variant sites, respectively, after filtration (Table S4). For P5 primary lung cancer and matched brain metastasis tissues, we identified 76 (in 27 genes) and 53 (in 19 genes) exonic somatic variant sites, respectively, after filtration (Table S5). For P6 primary tumor and matched brain metastasis samples, we identified 62 (in 29 genes) and 73 (in 34 genes) exonic somatic variant sites, respectively, after filtration (Table S6). Of these, more than 98% of the mutations were non-synonymous after filtering. Figure 1 shows a detailed summary of the genetic heterogeneity and homogeneity distribution for all mutations found in each patient.

**Functional prediction and bioinformatic analysis of brain metastasis-related genes**

Non-synonymous mutations refer to gene mutations that result in altered amino acid sequences of a polypeptide product or altered functional RNA sequences. Because the body has a complex mismatch repair mechanism, not all non-synonymous mutations are pathogenic or fatal. According to previous studies,
we applied SIFT, LRT and PolyPhen (polymorphism phe-
notyping) to further predict the impact of pathogenic somatic mutations on gene function [8,9]. We found that approximately 19.3% of the identified somatic mutations were likely to have pathogenic consequences according to at least two of the three widely accepted methods above. We included mutations annotated as ‘probably damaging’ or ‘possibly damaging’ or ‘deleterious’.

In order to better understand the function and pathway enrichment of the possible driver genes for brain metastases in NSCLC, we analyzed 661 commonalities and shared pathogenic genes of corresponding primary tumors and metastases using DAVID (Table S7). We found that the enriched GO BPs and MF were primarily associated with signal transduction and molecular binding (Tables S8–S10), including G-protein-coupled receptor signaling pathway ($n = 56$, $P = 1.82 \times 10^{-5}$, Fig. 2). We further used the KEGG pathway to examine the molecular interactions, reactions and relational networks of the shared pathogenic genes identified from primary tumors and paired-brain metastases. After classification, 206 genes were clustered into 126 pathways, of which 52 were associated with cancer-related signaling pathway (Table S11), including focal adhesion (COL6A3, FLNB, LAMC2, BAD, SHC1, LAMA2, TNR, and EGFR), ECM-receptor interactions (COL6A3, LAMC2, TNN, LAMA2, and TNR), cell cycle (CDC27, MCM2, MYT1, and EP300), the VEGF signaling pathway (PLA2G4A, BAD, and SPHK2), the Wnt signaling pathway (DAAM2, WNT10A, and EP300), the TP53 signaling pathway (BBC3 and BAII), the notch signaling pathway (NOTCH2, NOTCH2NL, MAML3, and EP300) and immunity-associated processes (CD2, HLA, CD80, NCR2, KLRC2, and SHC1).

**Genetic heterogeneity between primary tumors and brain metastases**

We compared six paired samples and observed genetic heterogeneity between primary tumor and corresponding brain metastases. Mutations unique to primary tumor or brain metastases may be acquired or lost during malignant progression, suggesting that there may be genetic alterations acquired during malignant
progression, possibly participating in BM in NSCLC. The LDHAL6B, CSH1, PEX5, and YBX2 genes were frequently altered in the primary tumors, as opposed to the brain metastases specimens. The SLC16A2, PLBD2, APC, ALPPL2, SCUBE2, OR8G5, and EVPL genes were only mutated in primary tumors but not in brain metastases.

Concordance of EGFR and TP53 mutations in primary tumors and brain metastases

Four patients had an activating mutation in EGFR (L858R or exon 19 deletions) in both the primary tumor and their corresponding BM. Three patients had deletions in exon 19, and one had a mutation in exon 21 (L858R). However, T790M was not detected in any of these patients with brain metastases. Additionally, we found that 75% of the patients in our cohort had mutations in TP53 (R43H, R16H, R136H, and R175H), but no discordance was found in these cases.

Of note, the concordance rate between primary NSCLC and brain metastases was 100% (Table 2). EGFR and TP53 status maybe homogeneous in lung...
cancer tissues and were stable during brain metastases during the malignant process.

**Hotspot mutations in brain-metastatic NSCLC patients**

We detected some well-known genes with high-frequency mutations in 12 tissue samples, including NOTCH2 (12/12), NOTCH2NL (12/12), FANCD2 (9/12), TP53 (8/12), and EGRF (8/12), followed by MSH2 (7/12), ATXN1 (7/12), and LRP1B (7/12). Of note, six patients with NOTCH2 mutations also had NOTCH2NL mutations. Figure 3 shows some of the most commonly altered cancer-related genes identified from each specimen.

**Discussion**

Lung cancer is a highly heterogeneous disease that varies in its degree of complexity and heterogeneity during the malignant process. Lung cancer is the most common cancer to metastasize to the brain [10]. Given the poor prognosis of brain-metastatic lung cancer, there is an urgent need to understand brain-metastatic genetic profiles by comparing the somatic mutations between primary tumors and corresponding brain metastases. Tumor metastasis is a complex and multi-stage process, consisting of local invasion, intravasation to the blood or lymphatic system, survival in the circulation, extravasation, and distant colonization [11]. Generally, cancer cells have difficulty penetrating the blood–brain barrier with its tight layer of endothelial cells. Nevertheless, lung cancer cells target and infiltrate the brain frequently. Considering this metastatic selective advantage, lung cancer cells may require highly specialized functions during infiltration into the brain parenchyma [12]. The genetic alterations driving brain organ-specific metastases remain unknown.

Previous efforts have been made to characterize the genomic heterogeneity of several target genes between primary lung cancer and brain metastases, but the driver mutant genetic profiles and underlying mechanisms of BM progress remain poorly understood. For example, Fan *et al.* [13] performed next-generation targeted panel sequencing of paired primary tumor and corresponding cerebrospinal fluid samples from 11 EGRF mutation-positive adenocarcinoma patients with leptomeningeal metastases. They showed a high degree of genetic heterogeneity and divergence within primary tumors and corresponding cerebrospinal fluid samples. Mansfield *et al.* [14] analyzed the expression of programmed cell death ligand 1 (PD-L1) in paired lung cancer and brain metastases from 73 patients using immunohistochemistry and showed that there was an agreement of PD-L1 expression in 86% of the paired specimens. However, Pinato *et al.*’s [15] and Zhou *et al.*’s [16] studies suggested a spatial and temporal heterogeneity in the expression of PD-L1 between isogenic NSCLC and corresponding distant metastases, and they believed that such heterogeneity was significantly dependent on whether BM was synchronously diagnosed along with primary disease.

Our data suggested that the detected SNVs and associated mutant genes varied markedly not only within paired primary tumor and corresponding brain metastases but also in different patients. Intratumor heterogeneity may be influenced by chromosome instability, and therefore metastasis may be driven via selection of cancer cells with SNV and may increase...
the risk of recurrence or death [17]. Genetic heterogeneity acquired during malignant progression highlights the challenge of exploring the mechanisms of 
BM. Additional genetic studies and functional assessments are needed to explore the contribution of mutant molecular profiles and their role in brain metastases.

Even though we observed high genetic heterogeneity in paired primary tumors and brain parenchyma metastases, we found that mutations in *EGFR* and *TP53* were highly concordant between paired primary tumors and brain metastases (100% concordance), the same as the concordance reported by Matsumoto *et al.* [18]. The majority of previous studies demonstrated that the overall concordance rate of *EGFR* mutation status was about 87.8% between primary tumor and paired metastatic lymph nodes [19]. A higher concordance rate was observed in Sun *et al.*’s [20] and Schmidt *et al.*’s [21] studies (91% and 94%, respectively). Yatabe *et al.* [22] reported no divergent *EGFR* mutations among 77 paired primary and matched lymph node metastases or among 54 primary and corresponding recurrent tumors using RT-PCR. Fan *et al.* [13] reported that *EGFR* mutations detected in the primary adenocarcinoma were highly concordant with those of matched cerebrospinal fluid samples from patients with brain metastases. Luo *et al.* [23] analyzed the *EGFR* mutational status in NSCLC patients and found a concordance rate of 93.3% between 15 paired primary and brain metastasis samples by an amplification mutation refractory system. Shimizu *et al.* [24] found that *EGFR* mutations detected in all metastatic sites were also detected in the corresponding primary tumors by the peptide nucleic acid-locked nucleic acid polymerase chain reaction, and that *EGFR* mutations in metastasis sites were sensitive markers of the response to targeted therapy in NSCLC patients. The slight discordance rate of mutations between paired primary tumors and metastases may be partly influenced by the testing technology [25] and sample quality [26]. Thus, our results agree with previously published data. The implication is that the stable *EGFR* mutational status between paired primary tumors and metastatic sites confirms that most mutations detected on analysis of the primary tumor or metastases are sufficient for clinical decision-making and that there is no need to re-biopsy recurrent tumors or metastases for most of the NSCLC patients.

*EGFR* mutations can be detected in more than 40% of the East Asian population with NSCLC [10]. In our experience, *EGFR*-activating mutations were detected in four of six patients, including L858R in one patient and exon 19 deletion in three patients. However, T790M was not detected in any of these patients with brain metastases. Additionally, *EGFR* was most frequently mutated in NSCLC, reported by COSMIC (approximately 27%). By comparing *EGFR* mutations with those reported in COSMIC, we identified a higher prevalence of *EGFR* mutations in patients with brain metastases. Similarly, Matsumoto *et al.* [18] reported *EGFR* mutations in 61% of lung adenocarcinoma patients with brain metastases. Based on previous studies and our group’s previous study, we conclude that patients with brain metastases harbor *EGFR* mutations more frequently than do those without, and that *EGFR* mutations are poor prognostic factors in stage IV patients with brain metastases [27].

Additionally, we found that 75% of the specimens in our cohort had mutations in *TP53*. However, the result is limited by the small number of patients; more studies and functional assessments are required. The concordance within the paired samples is also noteworthy, as *TP53* could be a potential therapeutic target for brain metastases. The well-known tumor suppressor gene *TP53* participates in inhibition of the propagation of cancer cells with genome instability [28]. Therefore, mutations in *TP53* may facilitate chromosome-unstable cell proliferation without regulation. Previous sequencing studies investigated *TP53* molecular characteristics in NSCLC within primary tumors and between primary tumors and metastases [29]. Multi-region WES was performed in 100 NSCLC patients, revealing that driver mutations in *EGFR* and *TP53* were almost exclusively clonal in different primary tumor regions from the same patients [17]. Other studies and reviews also showed that intratumoral heterogeneity of *TP53* mutations was rare in NSCLC and corresponding metastases [30]. We also identified a large number of *NOTCH2*, *NOTCH2NL*, and *FANCD2* mutations, much higher than the mutation rates reported by COSMIC in NSCLC (3%, 0.64%, and 0.94%, respectively). Of note, six patients with *NOTCH2* mutations also had *NOTCH2NL* mutations. However, the result is limited by the small number of patients. More studies and functional assessments are required.

**Conclusion**

We observed genetic heterogeneity not only between the primary tumors and metastases, but also in different patients. These results imply that most genetic alterations may be acquired or lost during malignant progression. However, mutations in *EGFR* and *TP53* remained clonal during the malignant process. Concordance mutations in *EGFR* and *TP53* suggest that
activated oncogene EGFR and disrupted tumor suppressor TP53 may contribute to brain metastases. EGFR and TP53 mutations detected from the primary tumor may be sufficient for clinical treatment decision-making, and additional EGFR and TP53 testing of brain metastases may be unnecessary.

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Author contributions

XZ and XL conceived the study design. MG, RH and QZ collected the human samples. LL and XJ performed the analysis. LL wrote the paper. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Mutant genes identified in P1 primary tumor and matched brain metastasis samples.
Table S2. Mutant genes identified in P2 primary tumor and matched brain metastasis samples.
Table S3. Mutant genes identified in P3 primary tumor and matched brain metastasis samples.
Table S4. Mutant genes identified in P4 primary tumor and matched brain metastasis samples.
Table S5. Mutant genes identified in P5 primary tumor and matched brain metastasis samples.
Table S6. Mutant genes identified in P6 primary tumors and matched brain metastasis samples.
Table S7. Shared pathogenic genes of six corresponding primary tumors and brain metastases (661 in total).
Table S8. The gene ontology biological processes (GO-BP) analysis of shared pathogenic genes of corresponding primary tumors and metastases.
Table S9. The gene ontology cellular component (GO-CC) analysis of shared pathogenic genes of corresponding primary tumors and metastases.
Table S10. The gene ontology molecular function (GO-MF) analysis of shared pathogenic genes of corresponding primary tumors and metastases.
Table S11. KEGG pathway analysis: 52 pathways associated with cancer-related signaling.