# Genetic Heterogeneity Between Paired Primary and Brain Metastases in Lung Adenocarcinoma

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## ABSTRACT

**PURPOSE:** About one-third of nonsmall cell lung cancer (NSCLC) patients develop brain metastases (BM). However, there is an unmet need for early diagnosis and treatment of BM. The precise mechanism for BM is still unknown. However, the genetic heterogeneity between primary tumor and paired BM indicates that sampling from the primary tumor may not be able to fully represent the mutational status in metastases. In this study, the genetic heterogeneity of primary lung adenocarcinoma and paired BM was analyzed.

**PATIENTS AND METHODS:** A total of 11 paired samples of primary tumors and BM from lung cancer patients were included, in which 7 paired samples of patients were finally analyzed. Samples were sequenced by whole-exome sequencing (WES) to investigate the common and unique mutations in the primary tumors and BM, and the similarities and differences in copy number variation (CNV).

**RESULTS:** The consistency of gene mutation between primary lung adenocarcinoma and paired BM was 33% to 86%. FAM129C and ADAMTSs specifically mutated in BM, along with NKX2-1 high amplification and SAMD2/4 copy number deletion.

**CONCLUSION:** The consistency of gene mutation between primary lung adenocarcinoma and corresponding BM is relatively high, while the individual differences were significant. FAM129C and ADAMTSs mutations and high amplification of NKX2-1 may be related to BM of lung cancer. The loss of copy number of SAMD2/4 may be a potential therapeutic target for BM from lung adenocarcinoma.

**KEYWORDS:** Lung adenocarcinoma, primary, brain metastases, heterogeneity

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## Introduction

Lung cancer is the most common malignant tumor with the highest morbidity and mortality, of which nonsmall cell lung cancer (NSCLC) accounts for 80% to 85%.[1] However, at least 57% of patients with NSCLC had metastasized at the time of diagnosis and missed the opportunity for surgery.[2] Brain metastases (BM) is a significant cause of death in patients with advanced malignancies, which occurs in about 20% of lung cancer.[3] Furthermore, most of them are manifested as multiple metastases.[4] Brain metastases from lung cancer seriously affect the prognosis of patients, with average survival period after BM only 6 to 11 months.[5] Moreover, the efficacy of chemotherapy is somehow limited due to their inefficient capabilities to cross the blood-brain barrier (BBB), leading to poor drug exposure in the brain.[6,7] Targeted therapy based on molecular typing has achieved great success in NSCLC; especially the treatment by epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) with EGFR-sensitive mutation since 2015.[1] Osimertinib for example, as a third-generation EGFR-TKI, exhibit improved BBB permeability and more potent activities.[8] Besides, in recent years, immunotherapy, such as programmed cell death protein 1 (PD-1) or programmed death-ligand 1 (PD-L1) inhibitors, has also showed significant efficacy in NSCLC patients with BM.[9] Of note, different characteristics of tumor microenvironments between primary tumor and BM, including tumor mutation burden, certain genetic variations, or T-cell infiltration, are reported to be associated with the efficacy of PD-1 or PD-L1 inhibitors.[10,11] Therefore, it is important to perform genetic testing on the molecular typing of NSCLC.

All tumors are derived from genetic variation, and the development of tumors is a process of constant accumulation of genetic and epigenetic variation.[12] Next-generation sequencing (NGS) of primary tumor tissue samples is widely accepted as a practical method to determine genetic variations of lung cancer patients.[13,14] However, the potential genetic heterogeneity between primary tumor and corresponding BM raises the question whether primary tumor sample can be an alternative for the detection of genetic variations in BM. As previously reported, genomic analyses of BM and matching primary tumor have revealed that BM can harbor unique potentially actionable driver mutations.[15] Herein, this study focused on the

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genetic heterogeneity between primary lung adenocarcinoma and BM to explore the specific variation of BM.

## Patients and Methods

### *Study population*

Patients included in this study were screened from patients admitted to Daping Hospital affiliated to the Army Medical University (Chongqing, China) from January 2010 to October 2013. All patients were diagnosed as lung adenocarcinoma with BM. Tumor samples obtained by surgery or puncture were validated to be lung adenocarcinoma by histopathology, and all of them were prepared into formalin-fixed paraffin-embedded (FFPE) tumor samples, meeting the requirements of NGS.

### *Methods*

*Ethical statement.* This study was approved by the ethics committee of Daping Hospital affiliated to Army Medical University, Chongqing, China (Ethics file no. 202061). The need for consent was waived by the ethics committee after evaluation of the study design.

*DNA extraction.* DNA from FFPE sections was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen) according to the standard protocol. Samples satisfied the following tests, including concentration, sample integrity, and purification, which were chosen for constructing the exome sequencing library.

*DNA library construction and NGS.* For each sample, the extracted genomic DNA was randomly fragmented by Covaris to an average size of 200 to 250 bp. After end-repaired and adaptors ligated, these fragments were amplified by polymerase chain reaction (PCR). Then we used the required amount of precapture library for whole-exome capture with Agilent SureSelect Human All Exon51M following the standard manufacturer's protocol. The final qualified libraries were used to amplify on cBot to generate the cluster on the flow cell, and the amplified flow cell was sequenced pair end on HiSeq 2000 System at 90 bp read length. The average sequence depth was 157× (range from 118× to 191×).

### *Bioinformatics analysis*

*Mapping.* After removing reads containing sequencing adapters and low-quality reads with more than 5 ambiguous, high-quality reads were aligned to the NCBI human reference (hg19) using BWA (v 0.5.9) with default parameters, and then we used Picard to mark duplicates.

*Somatic mutation.* Somatic point mutations were detected by VarScan2.2.5. Somatic indels were predicted with GATK Somatic Indel Detector with default parameters. All high-confident mutations were obtained using an in-house pipeline coupled with visual inspection and the mutations with variant frequency smaller than 0.1 were discarded. For the mutation that was only confidently detected in primary tumor or metastases but not in the other one, Samtools was applied to check if some reads could support this mutation. For the percent of support reads reach 2%, the mutation was considered to exist in both primary tumors and metastases. Then all called mutations were annotated with ANNOVAR and applied to DAVID pathway enrichment analysis.

*Mutation signatures.* To decipher the mutational process in lung cancer, the somatic mutations of our research was merged with mutations of 183 lung adenocarcinoma called by Imielinski et al[16] and delineated their mutational signatures using the method proposed by Alexandrov et al.[17]

*Copy number variation.* Adjacent healthy tissues were used as a control for copy number variation (CNV). Copy number analysis from whole-exome sequencing (WES) data was performed using ReCapSeg. To identify the difference of significantly amplified or deleted peaks between primary tumors and BM, the gistic 2 algorithm was utilized to analyze the segmentation data produced by ReCapSeg. The peak was determined as amplified or deleted significantly when $q$ value $<.25$. The copy number variants of drug target genes were extracted from the segment result of ReCapSeg, which provided the judgment of CNV amplification or deletion levels.

## Results

### *Patient characteristics and tumor specimens*

Due to the complicated acquisition of BM specimens, a total of 11 triples of primary tumors, adjacent normal tissues, and corresponding BM were collected, which were labeled as 1, 2, 3, 4, 6, 8, 9, 10, 11, 14, and 15 (Table 1). Among them, No. 6 was confirmed as lung squamous cell carcinoma, while No. 8, 9, and 11 only did not show enough mutations that could not be included in the analysis mainly due to unsatisfied quality of tumor tissue. Therefore, only 7 triples of detection results were qualified to be examined in the study.

### *The mutation landscape of alterations in primary and corresponding BM tumors*

Somatic mutational profiles of 7 lung adenocarcinoma patients with corresponding BM were aggregated for the WES. These samples exhibited 3003 somatic alterations in gene regions in which 1252 were shared by primary and BM tumors. On the other hand, 113 specific mutations were detected in primary tumors, while 386 unique mutations were observed in BM. Two distinct patterns with varying mutation burdens were identified, with 5 paired samples were regular-mutated (1.73 mutations/Mb; range, 1.06-2.38), and the remaining 2 paired samples showed a hyper-mutated pattern (sample 4 and 14, 19.13 mutations/Mb; range, 13.25-21.97; Figure 1A). Comparing somatic

**Table 1.** Patient characteristics.

| PATIENT | SEX | AGE | SMOKING STATUS | PRIMARY TUMOR HISTOLOGIC TYPE | T CATEGORY | N CATEGORY | SYNCHRONOUS BRAIN METASTASIS | TIME TO BRAIN METASTASIS (MONTHS) |
|---|---|---|---|---|---|---|---|---|
| 1 | F | 43 | No | Adenocarcinoma | 2 | 1 | No | 13 |
| 2 | M | 54 | Yes | Adenocarcinoma | 2 | 0 | No | 9 |
| 3 | F | 38 | No | Adenocarcinoma | 2 | 0 | Yes | – |
| 4 | M | 48 | No | Adenocarcinoma | 2 | 1 | No | 60 |
| 10 | M | 63 | Yes | Adenocarcinoma | 2 | 0 | Yes | – |
| 14 | M | 51 | Yes | Adenocarcinoma | 3 | 0 | No | 8 |
| 15 | F | 54 | No | Adenocarcinoma | 2 | 2 | Yes | – |

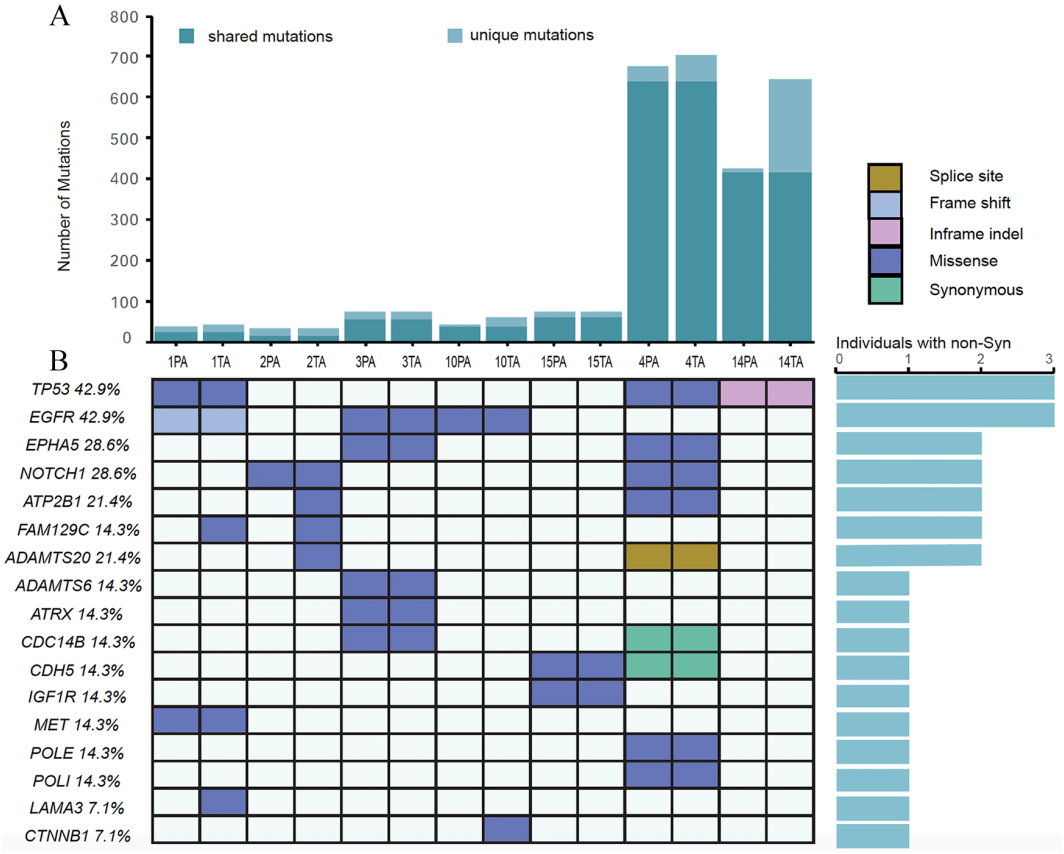


**Figure 1.** An overview of somatic mutations between corresponding primary tumors and brain metastases from 7 patients: (A) the correlation of somatic mutations in corresponding primary tumors and brain metastases and (B) mutations infrequently mutated genes or targetable genes with existing drug inhibitors—gene selection based on 1. Belong to the gene of the TARGET database, ruled out those only exist in no. 4 and no. 14 samples (EGFR, TP53, EPHA5, NOTCH1, IGF1R, ATRX, MET, and CTNNB1); 2. Mutated in 2 or more than 2 patients (ATP2B1, FAM129C, ADAMTS20, CDH5, CCDC14B, and PRKG2); 3. SIFT $<.05$ (TGFA and LAMA3) was found to be related to metastases, and the mutation was harmful. ADAMT6 was also selected for being from the same family as ADAMTS20. The hypermutation-related genes POLE, POLI are also listed separately, as they altered in hyper-mutated sample no. 4. EGFR indicates epidermal growth factor receptor.

mutations in primary tumors with their corresponding BM, the concordance rate is relatively high, which ranges from 33% to 86%. Description of alterations in primary and corresponding BM tumors was shown in Figure 1B. Several genes associated with DNA damage repair (DDR), were found in hyper-mutated samples, such as POLE, POLI, and MSH6, which may result in the rapidity accumulation of mutations.

TP53 and EGFR mutations were the most frequently observed alterations (42.9%) harbored by 3 patients. EPHA5 mutations were identified in 2 patients (28.6%). L858R mutation of EGFR, frequently detected in EGFR-mutated lung cancers,[1] was found in 2 cases (Patients 3 and 10), while frameshift alteration of EGFR was found in 1 case (Patient 1). NOTCH1 mutation, which was known to affect lung cancer

**Table 2.** Pathway analysis results of primary and metastatic samples.

| KEGG PATHWAY | NO.OF COEXIST | MUTATED GENE OF COEXIST | NO. OF SPECIFIC IN METASTASIS | MUTATED GENE OF METASTASIS |
|---|---|---|---|---|
| KEGG_FOCAL_ADHESION | 19 | *IGF1R,, LAMB2, MYLK,, COL11A1,ACTN4, RASGRF1,ITGA10,COL6A3,ITGA4,COL4A6, COL4A2,ITGB4,PDGFB, ACTN2, KDR,,* | 5 | *LAMA5, CTNNB1, PTEN,, LAMA3* |
| KEGG_WNT_SIGNALING_ PATHWAY | 7 | *PPP3R2, APC2,LEF1,CREBBP, DAAM2,VANGL2,TP53* | 5 | *CTBP2, DKK2, BTRC, PLCB4,CTNNB1* |
| KEGG_ PHOSPHATIDYLINOSITOL_ SIGNALING_SYSTEM | 5 | *DGKD, PIKFYVE, ITPR1,PIK3C2G,PIK3C2A* | 5 | *PLCB4,, PTEN,PIP4K2C,PLCG1* |
| KEGG_TIGHT_JUNCTION | 9 | *MYH15,, MYH8,MYH11,ACTN2,ACTN4,PPP 2R2B,EPB41L2, PARD3* | 4 | *MYH13, ASH1L, CTNNB1,PTEN* |
| KEGG_PROGESTERONE_ MEDIATED_OOCYTE_ MATURATION | 7 | *ADCY8, RPS6KA6,IGF1R,RPS6KA3,KRAS, HSP90AA1,CDC27* | 3 | *BRAF, MAD2L1, CDC23* |
| KEGG_REGULATION_OF_ ACTIN_CYTOSKELETON | 17 | *FGD3, TIAM1,KRAS,MYLK,ACTN4,ITGA10,I TGAM, ITGAD, ITGA4,APC2,PIKFYVE, ITGB4,ITGAX, CYFIP2,PDGFB,ACTN2,EGFR* | 2 | *PIP4K2C, BRAF* |
| KEGG_CALCIUM_ SIGNALING_PATHWAY | 14 | *PPP3R2, MYLK, GNAS, ATP2B1,GRM5,AD CY8,RYR1,RYR2,RYR3,GRIN2A,CACNA1C, EGFR,ITPR1,CACNA1I* | 2 | *PLCG1, PLCB4* |
| KEGG_CELL_ADHESION_ MOLECULES_CAMS | 9 | *CDH5, ITGAM,ITGA4,CD22,CD4,CNTN1,L1 CAM,CNTNAP2,CNTN2* | 2 | *CDH3, NEO1* |
| KEGG_GAP_JUNCTION | 9 | *GRM5, ADCY8,TUBB4B,EGFR,ITPR1,KRA S,GUCY1A2,PDGFB,GNAS* | 2 | *PLCB4, PRKG2* |
| KEGG_LEUKOCYTE_ TRANSENDOTHELIAL_ MIGRATION | 8 | *CDH5, ITGAM,ITGA4,SIPA1,MMP9,NCF2,A CTN2,ACTN4* | 2 | *PLCG1, CTNNB1* |
| KEGG_NOTCH_ SIGNALING_PATHWAY | 6 | *NOTCH1, CREBBP,JAG2,LFNG, NCSTN,* | 2 | *MAML1, CTBP2* |

Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genomes.

development,[18,19] was observed in 2 cases (28.6%). In the screening of specific mutant genes of BM (Figure 1), samples no. 4 and no. 14 were excluded since both samples are hypermutated, which exhibited interference to the overall mutation frequency statistics. Finally, 2 genes, FAM129C and ADAMTSs, were found specifically correlated with BM.

In pathway enrichment analysis, we observed that focal adhesion and extracellular matrix (ECM)-receptor interaction are the top 2 significant signaling pathways, which are essential for the formation of the BBB. Moreover, most genes belonging to these 2 pathways were mutated in both primary tumors and BM. The pathway analysis results of primary tumors and BM samples were detailed in Table 2. The enriched pathways in primary tumors and BM samples are similar, but the genes involved in those pathways are different.

### *Divergence of mutation signatures of primary and BM tumors*

To gain further insights into the mutations in primary tumors and BM, we delineated their mutation signatures using the computational framework proposed by Alexandrov et al.[17] Three mutation signatures, named signatures 1, 2, and 3, were extracted from the 7 patients in the current and 183 lung adenocarcinoma patients reported by Imielinski et al.[16] Each of them contributed to the different proportions of mutations in the primary and BM tumors. The mutation signature pattern was illustrated in Figure 2. Signature 1 is related to guanine's benzopyrene adduct, and signature 3 is featured with CG > TG. It is noted that signature 2, known as APOBEC signature widespread across multiple cancer types and associated with carcinogens-induced single-strand DNA breakage,[20] accounts for 13.59% mutations in the primary tumor while 22.49% in corresponding BM.

### *The significant discrepancy in focal copy number alterations was detected in obtained samples from the primary and corresponding BM tumors*

The genetic divergence of focal copy number between clinically sampled primary tumors and BM were detected to address their heterogeneity. A description of focal copy number variants is provided in Figure 3A. Focal amplification peaks of copy number alterations in primary tumors and BM were

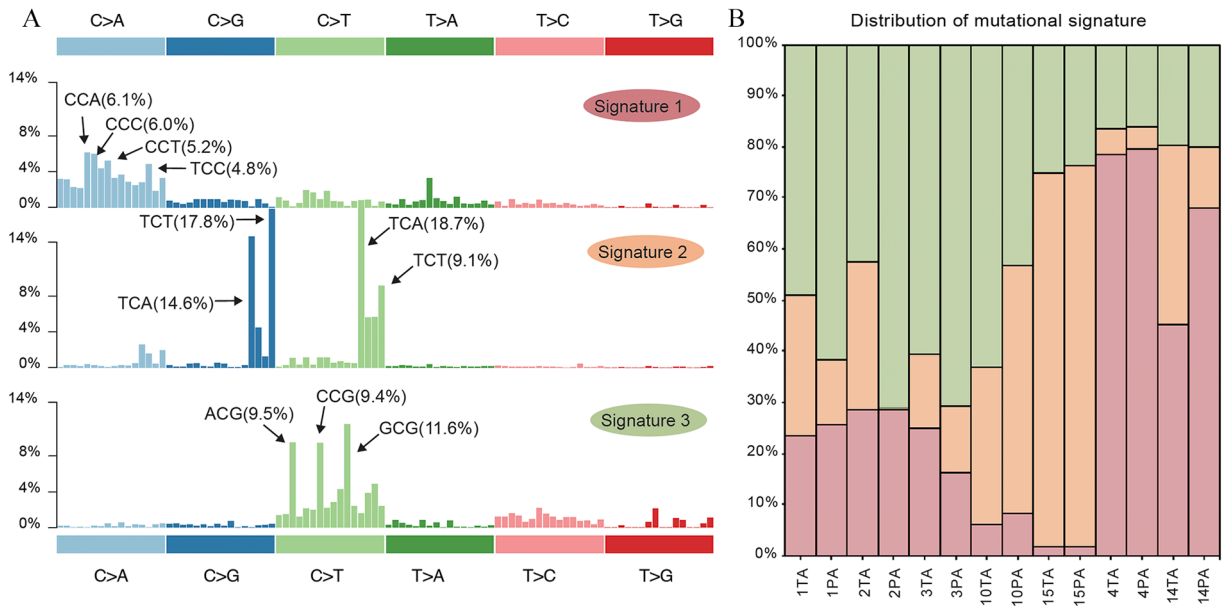


**Figure 2.** Mutational signature analysis of primary tumors and brain metastases: (A) identifying 3 mutational signatures from primary tumors, corresponding brain metastases, and 183 lung adenocarcinomas and (B) the contributions of 3 mutational signatures to 14 samples.

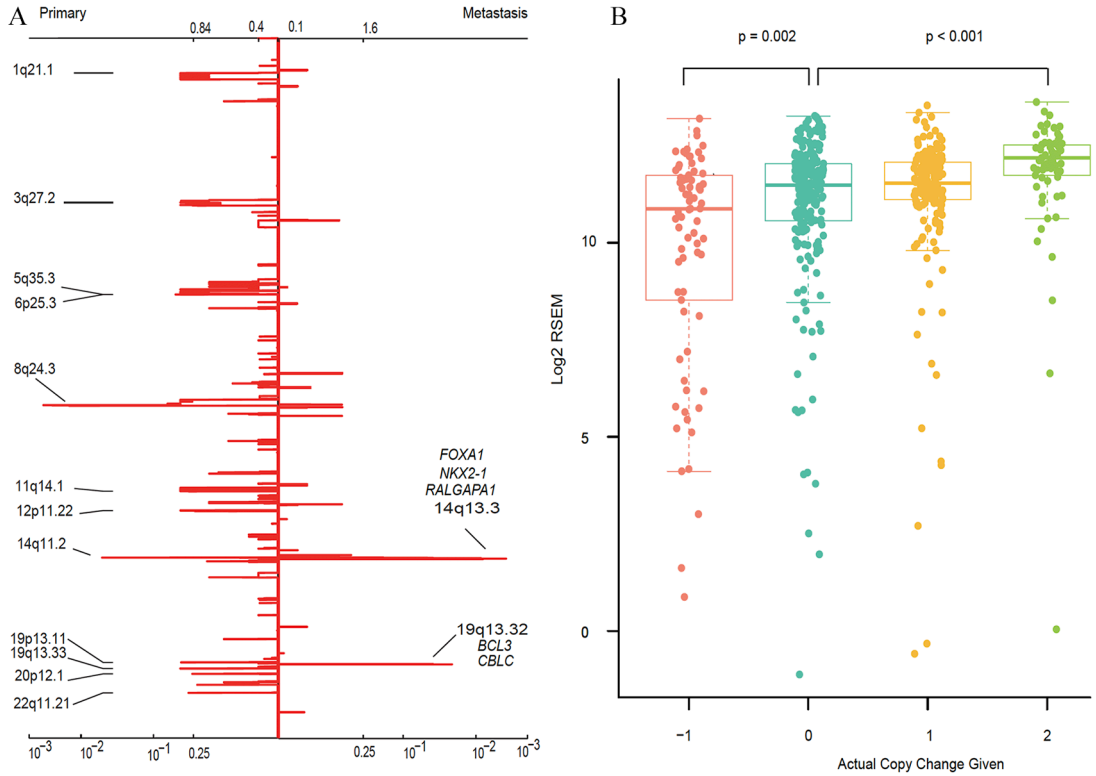


**Figure 3.** The characteristic of focal copy number alterations (A). Focal amplification peaks of copy number alterations in primary tumors and brain metastases; (B) the correlation between NKX2-1 actual copy number changes and expression levels in 514 LUAD samples from the TCGA data set. TCGA indicates The Cancer Genome Atlas.

different, especially with 2 significant extended peaks at 8q24.3 and 14q11.2 in the primary tumor, and 14q13.3 and 19q13.32 in BM. Of note, 5 reported genes (FOXA [57.1%, 4/7], NKX2-1 [57.1%, 4/7], RALGAPA1 [57.1%, 4/7], BCL3 [42.9%, 3/7], and CBLC [42.9%, 3/7]) exhibited high alteration prevalence in metastatic cancer. To gain further insights into the relationship between CNV and expression of NKX2-1, we obtained NKX2-1 expression profiles of 237 lung adenocarcinoma patients and corresponding copy number variants data from The Cancer Genome Atlas (TCGA) database. The corresponding expression pattern of NKX2-1 was calculated by RSEM analysis. We found that the group with NKX2-1

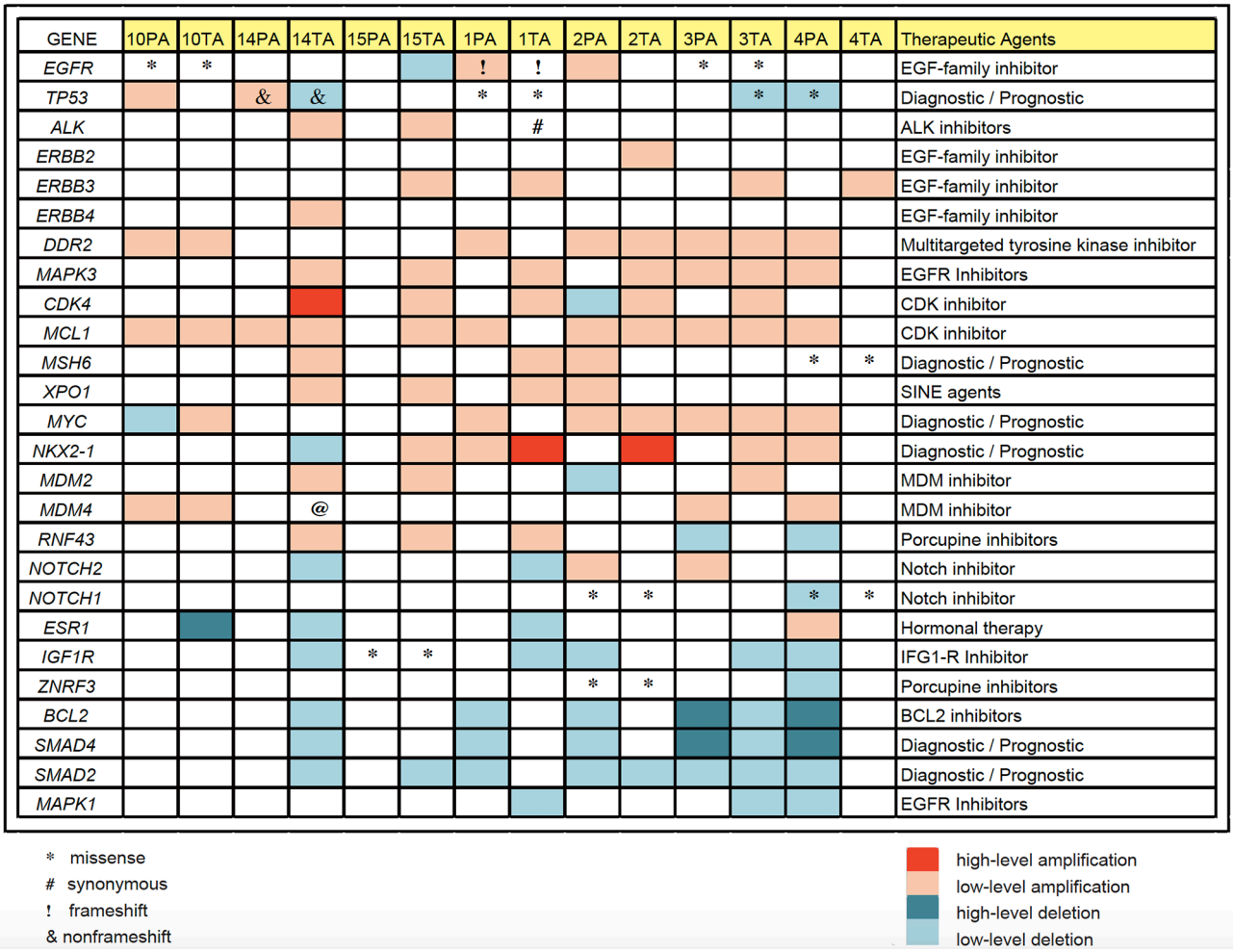


**Figure 4.** A landscape of copy number variants and somatic mutations of targetable genes in primary tumors and brain metastases.

amplification has significantly higher expression than the group with normal NKX2-1 copy numbers (Student *t*-test, $P < .001$), indicating that NKX2-1 amplification is more inclined to high expression.

Next, we compared somatic mutations and CNV of genes from the TARGET database. It is noted that ERBB2, ERBB3, and ERBB4 were merely amplified in metastases (Figure 4), while SMAD2 and SMAD4 showed copy number deletions both in primary and metastases tumors, which may act as tumor suppressors.

## Discussion

It has been suggested that BM develop in nearly 20% of individuals with lung cancer.[3] Pulmonary blood can flow to the brain directly, which may be associated with the frequent occurrence of BM from primary lung cancer.[21] Other factors include the genetic status of the tumor, BBB, tumor immune microenvironment, as well as immune recognition.[22] However, the specific mechanism of BM is still not fully clear.[21] Therefore, there is an unmet need for investigating mechanisms for BM.

Given the spatial heterogeneity of a tumor, the gene mutation status of a few tumor cells in the primary site may not represent the mutation status of distant metastases, including BM.[23,24] Genetic comparison of primary tumor and extracranial metastases with matched intracerebral metastases revealed potentially targetable mutations in BM which were not present in extracranial diseases, indicating that these mutations may not be detected from a single extracranial sample.[25,26] Indeed, more than 50% of BM harbor targetable alterations not detected in the primary tumor.[25] Therefore, studies on the heterogeneity, especially the genes with specific variations in the BM, will help clarify the mechanism of BM.

In this study, 7 triples samples of primary tumors, adjacent normal tissues, and corresponding BM tumors were analyzed. The analysis mainly focused on single-nucleotide variation (SNV), insertion, deletion, and CNV. The results show that the consistency rate between the 2 pairs is relatively high (33%-86%; Table 3), while the number of mutations is consistent with the consistency rate. Compared with the study reported by Vignot et al,[27] the consistency rate found in our study is significantly lower, possibly because Vignot et al adopted the targeted NGS assay method and only detected the limited range of 3230 exons in 182-cancer-related genes plus 37 introns from 14 genes. In our study, the WES method was used to detect more than 20 000 exons, which could better reflect the differences. In another study reported by Wang

**Table 3.** The consistency of gene mutation between primary and BM lesions.

| SAMPLE ID | NO. OF PRIMARY MUTATION | SHARED MUTATION | NO. OF METASTASIS MUTATION | CONCORDANCE RATE (%) |
|---|---|---|---|---|
| 1 | 17 | 23 | 21 | 37.70492 |
| 2 | 17 | 17 | 17 | 33.33333 |
| 3 | 19 | 57 | 19 | 60 |
| 4 | 36 | 639 | 64 | 86.4682 |
| 10 | 4 | 37 | 23 | 57.8125 |
| 14 | 6 | 418 | 229 | 64.01225 |
| 15 | 14 | 61 | 13 | 69.31818 |

Abbreviation: BM, brain metastases.

et al,[28] mutations of major drivers, including EGFR, KRAS, TP53, and ALK, were highly concordant between primary NSCLC and corresponding BM (>80%).

In this study, 2 genes of FAM129C and ADAMTSs were found possibly correlated with BM. FAM129C, also known as BCNP1, was shown to be involved in cancer, in that its phosphorylation is dependent on PI3K and p38MAPK and its degradation depending on a proteasome-mediated pathway.[29] ADAMTSs codes for extracellular protease, which can affect tumor microenvironment through multiple mechanisms and interact with other components or regulatory factors to affect cell adhesion, migration, proliferation, and angiogenesis.[30] In a study reported by Liao in 2018,[31] the LDHAL6B, CSH1, PEX5, and YBX2 genes were found to be frequently altered in the primary tumors, while SLC16A2, PLBD2, APC, ALPPL2, SCUBE2, OR8G5, EVPL genes were only mutated in primary tumors but not in BM. In addition, we found SAMD2 and SMAD4 showed copy number deletions in both primary and metastases tumors. These 2 genes were associated with the tumour growth factor (TGF)-beta signaling pathway,[32] and it has been reported that the TGF-beta signaling pathway is related to BM.[33] Besides, TP53 and EGFR mutations were observed in both primary and metastatic lesions, which is consistent with a previous study.[31] In the future, more mutations may be found based on new techniques, such as ctDNA detection of cerebrospinal fluid (CSF), which has attracted much attention because it may also display the genetic information of BM and have therapeutic indications.[34]

This study has several limitations. First, based on limited samples and clinical information, it is difficult to draw a "cause-effect" relationship between the new genes found and the risk of developing BM. In the future, with more samples enrolled, a logistic regression analysis along with extensive time course analysis may provide more evidence to determine the risk of BM development. Second, transgenic mouse model experiments are needed in the future to provide evidence that mutations of specific genes can result in BM.

In conclusion, this study found that the mutation consistency between the primary tumor tissue and the BM tissue was relatively high, but the differences between individuals were large. The mutation of FAM129C and ADAMTSs and the high amplification of NKX2-1 may be related to BM of lung cancer. The loss of copy number of SAMD2 and SMAD4 may be a therapeutic target for BM of lung cancer. The mechanism of BM in lung cancer needs to be elucidated by further investigation.

## Author Contributions

HY conceived and designed the study, HY, Li Li, and LZ drafted the paper and did the statistical analysis. HR, Li Lin, WM and HD collected the data.

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