Targeted exome sequencing for the identification of common mutational signatures and potential driver mutations for brain metastases and prognosis

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Abstract. Brain metastases (BMs) are malignancies in the central nervous system with poor prognosis. Genetic landscapes of the primary tumor sites have been extensively profiled; however, mutations associated with BMs are poorly understood. In the present study, target exome sequencing of 560 cancer-associated genes in samples from 52 patients with brain metastasis from various primary sites was performed. Recurrent mutations for BMs from distinct origins were identified. There were both genetic homogeneity and heterogeneity between BMs and primary lung tumor tissues. The mutation rate of the major cancer driver gene, *TP53*, was consistently high in both the primary lung cancer sites and BMs, while some genetic alterations, associated with DNA damage response deficiency, were specifically enriched in BMs. The mutational signatures enriched in BMs could serve as actionable targets for treatment. The mutation in the primary site of the potential brain metastasis driver gene, nuclear mitotic apparatus protein 1 (*NUMA1*), affected the progression-free survival time of patients with lung cancer, and patients with the *NUMA1* mutation in BMs had a good prognosis. This suggested that the occurrence and clinical outcome of brain metastases could be independent of each other.

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Abbreviations: BM, brain metastasis; TCGA, The Cancer Genome Atlas; SNV, single nucleotide variation

Key words: brain metastasis, targeted exome sequencing, brain metastasis-enriched mutations

Introduction

Brain metastases (BMs) are the most common malignancies in the central nervous system, and mostly migrate from lung cancer, melanoma and breast cancer (1). Brain metastasis is a pathological feature associated with poor prognosis (1). Cancer genomics has expanded the knowledge of driver mutations for various types of cancer, and has identified potential therapeutic targets and precise therapies over the past few decades. However, therapeutic approaches for BMs are restricted to surgical resection, whole brain radiotherapy and chemotherapy (2). Since traditional therapies are insufficient to improve the prognosis of BMs (1), there the identification of key molecular events mediating metastasis is an urgent requirement. Little is known regarding driver genomic alterations in BMs and to what extent brain metastasis samples share common mutations, which limits the mechanistic understanding and discovery of drug targets specifically for patients with brain metastasis.

A previous study has evaluated the genetic heterogeneity among the primary tumor site, paired normal tissue and BMs in a limited number of patients (3). However, it is still unclear to what extent different types of primary sites of BMs share common driver mutations or metastasis mechanisms. Extensive heterogeneity between primary sites and metastasis sites, and between spatially distinct metastasis sites have been observed in other types of cancer, including renal cell carcinoma (4). Several small-scale genomic profiling studies have revealed genetic alterations in patients with brain metastasis (5-7). Nearly half of the patients with lung cancer develop brain metastasis in the later stages of the disease (8), and alterations in the PI3K signaling pathway have been identified to mediate the formation of BMs in these patients (5,6). BMs from colorectal cancer are rare; however, they can cause

severe outcomes, and genomic profiling has suggested that deficiency in the DNA damage response is involved in the formation of BMs from colorectal cancer (7). There remains a requirement for comprehensive evaluation of homogeneity and heterogeneity between primary tumor sites and BMs, as well as between BMs from various primary tumor sites.

To gain a global view of brain metastasis heterogeneity and potential driver genes, targeted next-generation sequencing of 560 cancer-related genes in brain metastasis samples from various primary sites, with an emphasis on lung cancer, was performed in the present study. Further analysis of the mutational profiles provided insights into the clinical outcomes associated with genetic mutations enriched in BMs, suggesting that brain metastasis-related gene mutations are associated with poor prognosis.

Materials and methods

Patients, sample collection and follow-up survey. The present study obtained the records and samples of a total of 52 patients who underwent resection surgery for brain metastasis at Beijing Tiantan Hospital (Beijing, China). The median patient age was 57 years (age range, 36-73 years), 59.6% (31) were men and 40.4% (21) were women. The sequencing data were generated using tumors resected between February 2012 and January 2016. All samples were collected and frozen in liquid nitrogen within 5 min after resection, and were subjected to sequencing analysis. The survival status of the patients was obtained through phone contact every 3 months as a follow-up survey August 2018.

Library preparation and sequencing. The sequencing library was generated using 1 μg DNA per sample according to the guide of the Truseq Nano DNA HT Sample Prep Kit (Illumina, Inc.) with index codes added to each sample. The quality of genomic DNA was monitored on a 1% agarose gel, while the concentration was measured using the Qubit® DNA Assay Kit and Qubit® 2.0 Flurometer (Invitrogen; Thermo Fisher Scientific, Inc.). DNA sequencing was performed for all the exons of 559 cancer-related genes and the promoter of telomerase reverse transcriptase (Agilent SureSelect custom kit; Agilent Technologies, Inc.). Briefly, fragmentation was performed using a hydrodynamic shearing system (Covaris, Inc.) to generate 180-280 bp fragments. Extracted DNA was then amplified by ligation-mediated PCR (LM-PCR) using Herculase II Fusion DNA Polymerase and customized primer provided by the Agilent SureSelect custom kit (cat. no. G9611B; Agilent Technologies, Inc.), purified and hybridized to the probe for enrichment. The following thermocycling conditions were used: 98˚C for 2 min; 6 cycles at {98˚C for 30 sec, 65˚C for 30 sec and 72˚C for 1 min}, and 72˚C for 2 min. Non-hybridized fragments were subsequently washed using nuclease free water. Both non-captured and captured LM-PCR products were subjected to quantitative PCR to estimate the magnitude of enrichment using the KAPA Library Quantification kit (cat. no. KK4824; Kapa Biosystems. Inc.). The primer sequences used are as follows: Primer P1 5'-AAT GATACGGCGACCACCGA-3' and Primer P2: 5'-CAAGCA GAAGACGGCATACGA-3'. SYBR-Green I dye was used in the qPCR analysis and library quantification DNA standards 1-6 (a 10-fold dilution series of a linear, 452 bp template) were used as the reference for absolute quantification. The thermocycling conditions were as follows: 95˚C for 5 min for initial activation/denaturation, and 35 cycles denaturation, annealing and extension at 95˚C for 30 sec, and 60˚C for 45 sec. The DNA libraries were sequenced on the Illumina Hiseq 4000 platform (Illumina, Inc.), and 150-bp paired-end reads were generated at a depth of 1000X.

Detection and filtering of genomic alterations. Sequencing data were mapped to the human reference genome (UCSC hg19) using the Burrows-Wheeler Aligner software (version 0.7.10-r789) (9). SAMtools (version 0.1.19) was used to sort the BAM files and perform duplicate marking, local realignment and base quality recalibration to generate the final BAM file for computing the sequence coverage and depth (10). To identify single nucleotide variations (SNVs) and small insertions and deletions (InDels) from the BM samples, GATK (https://gatk.broadinstitute.org/hc/en-us) and SAMtools were used. In addition to default filters, polymorphisms of SNVs and InDels referenced in the 1000 Genomes Project (11), Exome Aggregation Consortium (12) or the in-house Novozhonghua database (not publicly available yet) with a minor allele frequency >1% were removed. Subsequently, the variant call format result was annotated by ANNOVAR (version 20191024) (13). The mutation frequency of the primary lung tumor site was obtained from a previous lung pan-cancer dataset through the cBio cancer genomics portal (https://www.cbioportal.org/) (14).

Statistical analysis. Survival analysis was performed using the R (v3.6.0) survival package (v3.2, https://cran.r-project.org/web/packages/survival/index.html). The overall survival rate was estimated according to the Kaplan-Meier method using the survfit function in the R survival package. A log-rank test was performed for comparison of survival curves using the survdiff function. Survival analysis was performed on 48 of the 52 patients with BM (four patients were excluded in the survival analysis as their dates of death were not accurately obtained). For each BM enriched mutated gene, patients were grouped according to whether they harbored the mutation or not. Survival analysis was performed between the two groups to identify genes whose mutation affects the overall survival of patients with BM. $P<0.05$ was set as the cutoff of significant differential overall survival rate in the log-rant test. Survival and progression-free survival analyses of the patients with lung cancer were performed using data from datasets and tools in the cBio cancer genomics portal (https://www.cbioportal.org) (14). To investigate whether BM mutation in the primary tumor affects the PFS of patients with lung cancer, the PFS of patients with or without BM enriched mutations in the datasets of 1,410 patients combined in cBio cancer genomics portal was assessed.

Results

Recurrent mutations among BMs. To identify genomic alterations associated with brain metastasis, targeted exome sequencing of a panel of 560 cancer-related genes was performed (Table SI). Exomes of these genes were targeted

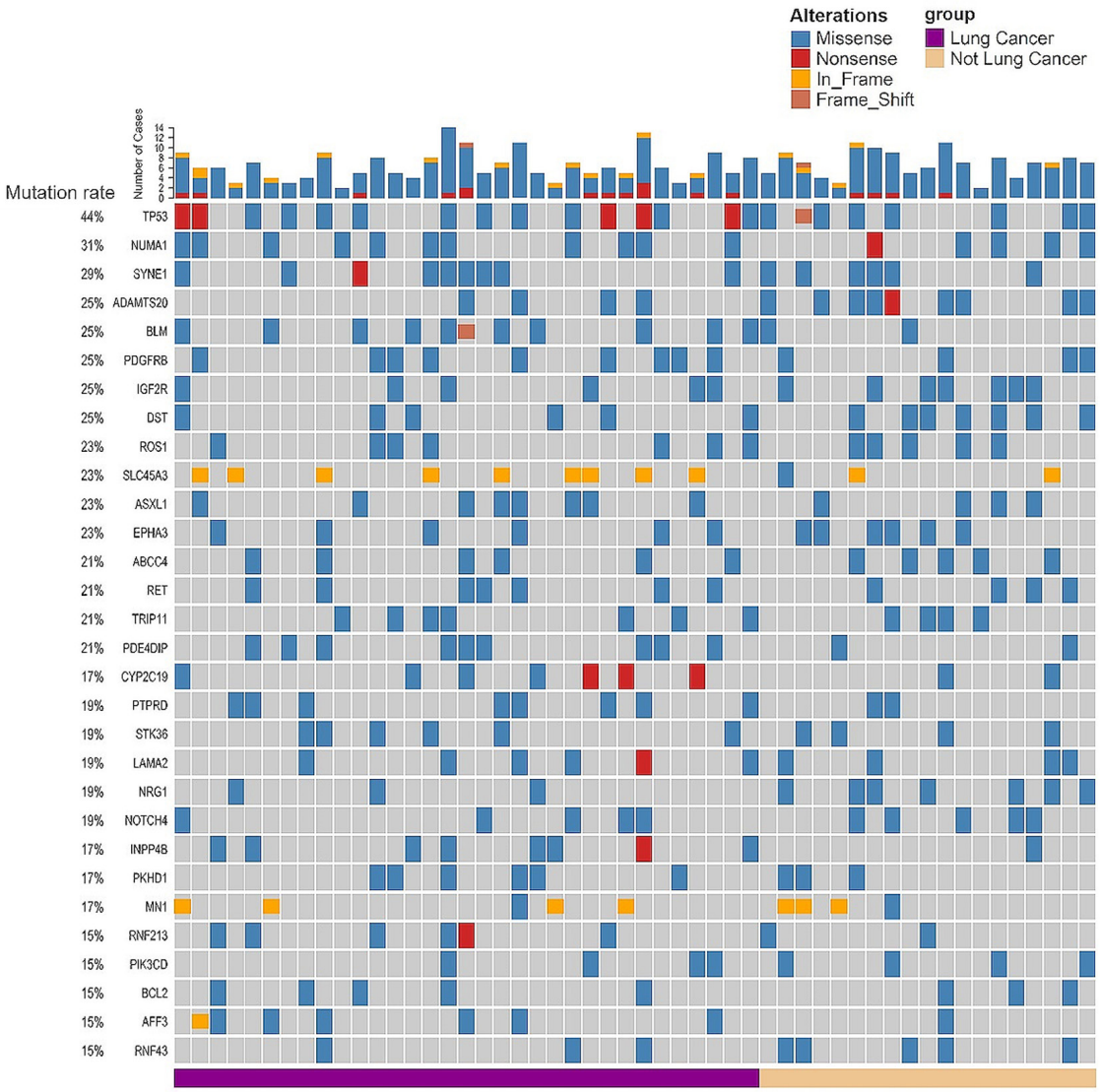

Figure 1. Landscape of the recurrent exome alterations in 52 brain metastases samples. Genes with a mutation rate >15% are shown. The mutation rate of each gene is shown on the left. The number of cases with the genetic mutations is shown at the top of the figure.

with the exception of TERT, whose promoter region was targeted using samples from 52 patients with brain metastases from various primary sites. Clinical characteristics of the patients are shown in Table SII. A total of 33 patients had primary lung cancer, while the remaining 19 patients had cancer of other primary locations. Recurrent mutations in these brain metastasis samples were identified by comparing the sequencing results of the targeted sites with the human reference genome. *TP53* was the most commonly mutated gene (44.2%) among all the brain metastasis samples, followed by other genes that have been frequently associated with cancer, including nuclear mitotic apparatus protein 1 (*NUMA1*), *SYNE1*, *PKHD1*, *ADAMTS20*, *BLM*, *PDGFRB*, *IGF2R* and *PKHD1* (Fig. 1).

Genomic alterations in BM-related genes (e.g. *SCN7A*, *SCN5A*, *SCN2A*, *IKZF1*, *PDZRN4* and *TP53*) have been identified in BMs from various primary sites (5-7,15); however, it remains unclear to what extent BMs from different sites have common and specific mutational signatures. Since the lungs were the primary site for 63.5% of the brain metastasis samples (Table SII), mutations which were common for all brain metastasis samples were investigated, as well as those that were more frequent in brain metastasis originating from the lungs. The frequency of recurrent mutations in brain metastasis from the lungs was compared with that of other sites (Fig. 2A and B). Among the 25 genes whose mutation frequency reached 15% in the brain metastasis samples from the lungs, 9 genes (*BLM*, *PDE4DIP*, *INPP4B*, *PTRRD*, *AFF3*, *HIF1A*,*CYP2C19*, *ARID1A* and *TGM7*; Fig. 2C; Table SIII) harbored a >2-fold mutation rate compared with that in brain metastasis samples from other primary tumor sites. Recurrent mutated genes, with a similar high mutation rate for different primary sites included *TP53*, *NUMA1*, *SYNE1*, *ASXL1*, *RET*, *ROS1* and *TRIP11*. There was no brain metastasis mutation identified that was exclusively found in patients with lung cancer, suggesting a limited effect of the tissue of origin on the brain metastasis genomic signature. The heterogeneity of brain metastasis genomic signatures was further supported by hierarchical clustering of the mutation signature of all brain metastasis samples (Fig. 2D).

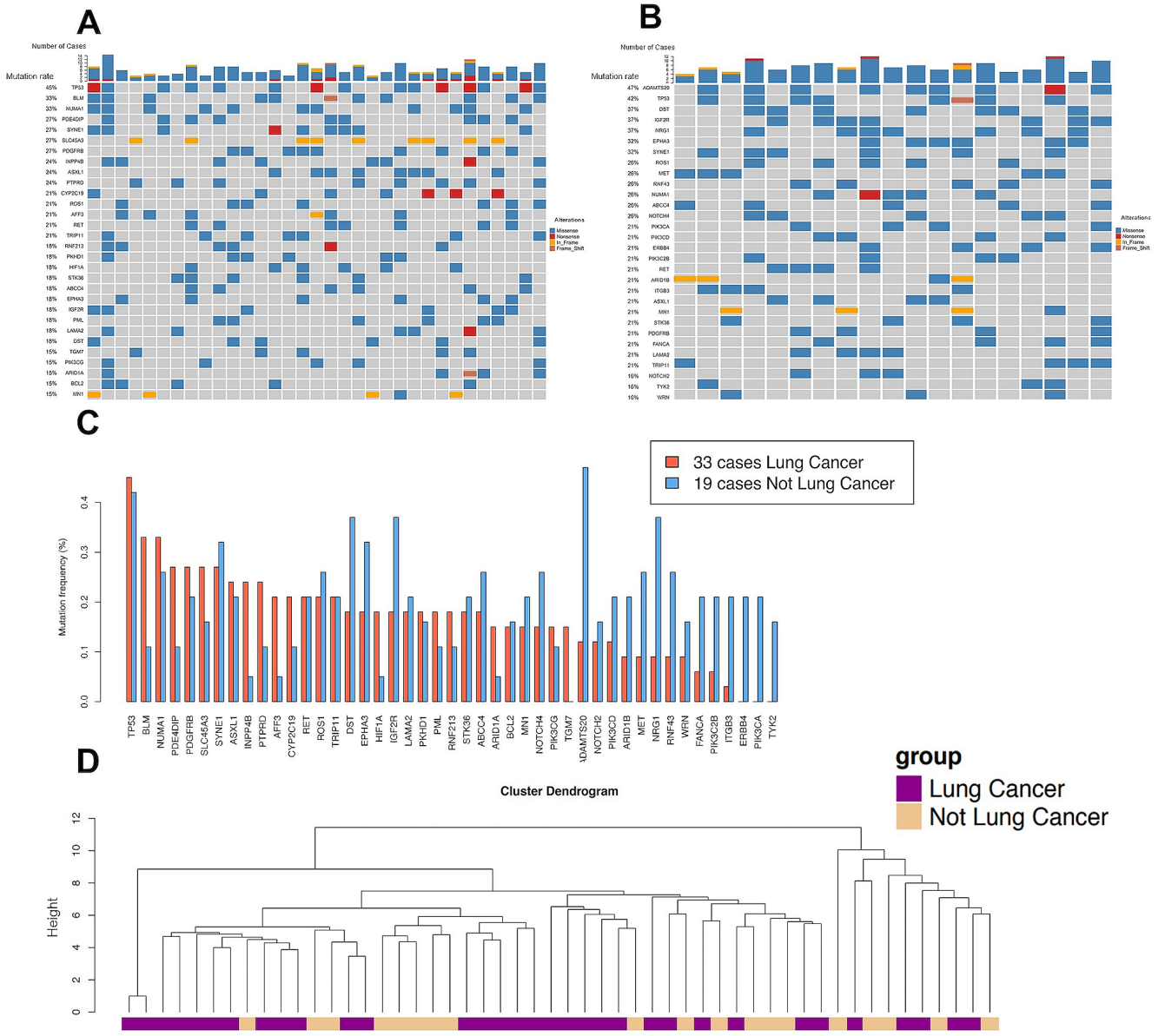

Figure 2. Exome alterations in BMs originating from the lungs and other primary sites. Landscape of the recurrent exome alterations in (A) 33 brain metastasis samples whose primary sites were the lungs, and (B) in 19 brain metastasis samples whose primary sites were not the lungs. (C) Mutation frequency in BMs samples from the lungs compared with those from other types of cancer. (D) Hierarchical clustering of mutational signatures in all brain metastasis samples revealed genetic heterogeneity of BMs. BMs, brain metastases.

Genomic alterations enriched in brain metastasis samples compared with in tumor samples from the lungs. To identify potential driver mutations that were enriched in the brain metastasis samples compared with in the primary site, the frequency of the recurrent gene mutations in the brain metastasis samples and the primary site was compared. *TP53* was the most frequently mutated gene in both the brain metastasis samples and tumors in the lungs, according to data from the present study and from a The Cancer Genome Atlas (TCGA) dataset, respectively (Fig. 3A). A total of two genes (*BLM* and *NUMA1*) were associated with DNA damage response (16,17), and had a >30% mutation rate in brain metastasis samples which had migrated from the lungs, compared with 2-3% in the lungs, as the primary site, suggesting that brain metastasis was associated with dysregulated DNA damage response. Brain metastasis has been associated with poor prognosis in patients with lung cancer (1,5). Therefore, to examine the potential link between brain metastasis-enriched mutated genes and clinical outcome, a published dataset of lung cancer genome sequencing was investigated (14,18-21). The mutation rates for potential brain metastasis driver genes such as BLM, NUMA1, SLC45A3 and PDGFRB were low in the primary site (Fig. 3A); however, a mutation in *NUMA1* in the primary lung cancer site was associated with worse progression-free survival time [35.6 months (n=69) vs. 67.2 months (n=1,341); log-rank test, P<0.01; Fig. 3B]. Furthermore, the *NUMA1* mutation did not affect the overall survival rate in patients with lung cancer (log-rank test, P=0.567; Fig. 3C). This result suggested that the *NUMA1* mutation may promote brain metastasis without affecting the overall survival time of patients with lung cancer. BMs migrated from the breasts and the colon have been demonstrated to be enriched with mutations and abnormal

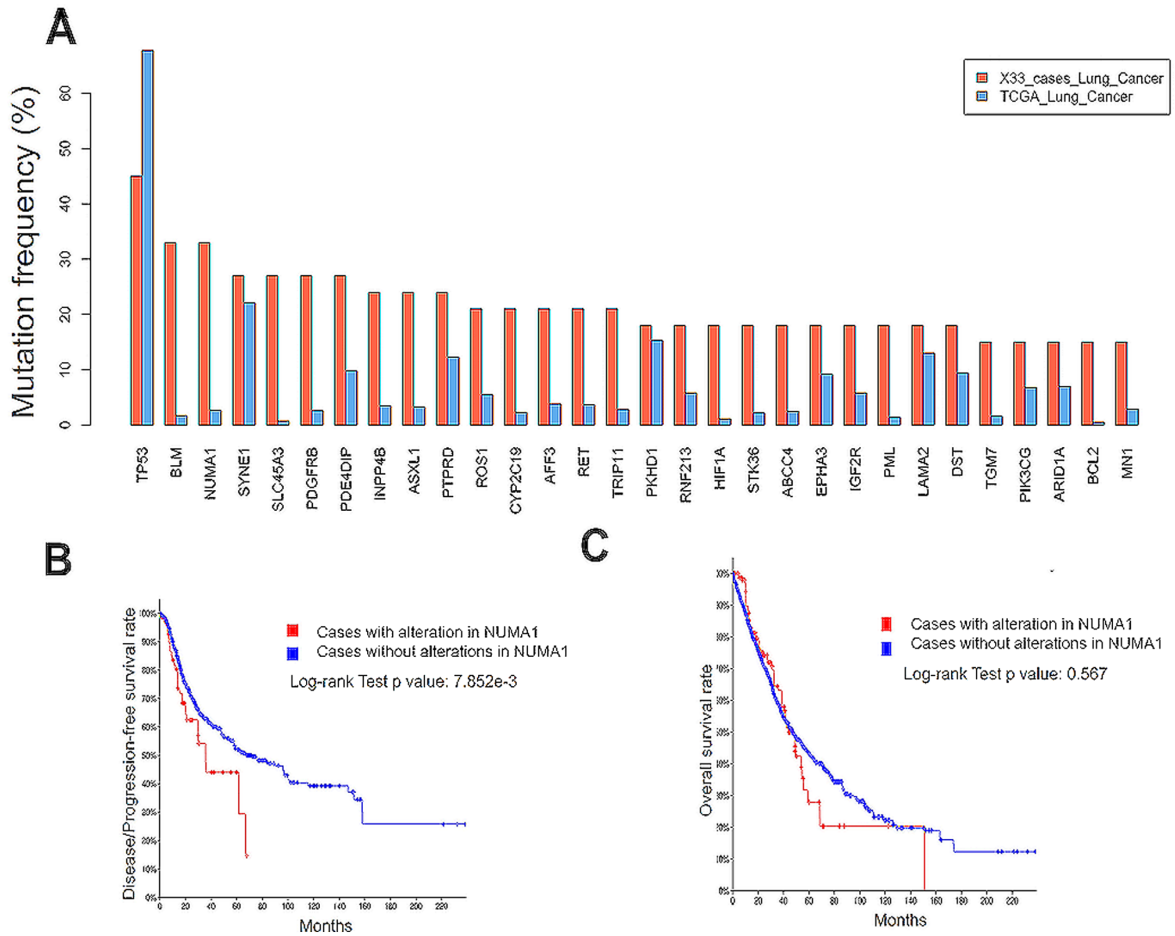

Figure 3. Brain metastasis-enriched mutations in patients with lung cancer. (A) Gene mutation frequency of brain metastases samples and the primary sites in patients with lung cancer. (B) Progression-free survival time of patients with lung cancer with or without the *NUMA1* mutation. (C) Overall survival time of patients with lung cancer with or without the *NUMA1* mutation. *NUMA1*, nuclear mitotic apparatus protein 1; TCGA, The Cancer Genome Atlas.

expression levels of DNA damage repair genes (7,22,23). The results of the present study suggested that DNA damage repair deficiency was a common feature of BMs as genes related to DNA damage responses (BLM and NUMA1) was frequently mutated in BMs from various primary sties.

Common genetic alterations in BMs and prognosis. A total of 8 genes with recurrent mutations in both BMs from the lungs and other primary sites in at least 9 patients were identified (Fig. 1). Significant prognostic markers for brain metastasis samples were rarely identified previously (1,5,6,8). To investigate gene mutations associated with prognosis in patients with brain metastasis, the overall survival rate of patients with or without these mutations was investigated. Most genetic alterations in BMs were not associated with the overall survival rate; however, a mutation in the potential brain metastasis driver gene, *NUMA1*, could predict a good prognosis in patients with brain metastasis (Fig. 4A), suggesting *NUMA1* may be a potential prognostic marker for brain metastasis progression. The frequency of brain metastasis for different types of cancer varies greatly; however, the clinical outcome of patients with brain metastasis and different primary sites is unknown. It was identified that patients with lung cancer were more prone to have brain metastasis; however, they had a good prognosis compared with patients with BMs that had migrated from other sites (Fig. 4B). These results suggested that the clinical outcome of BMs may not be associated with the frequency of brain metastasis formation.

Discussion

The prognosis of patients with brain metastasis is poor, with a median survival time of a few months (1). The incidence of brain metastasis is rising, as revolutionized cancer therapy has improved the survival of patients with advanced cancer (24). In contrast to the advancement of treatment of primary tumors, the treatment of brain metastasis remains a substantial challenge, primarily due to the lack of actionable targets (1,2,8). Previous high-throughput sequencing studies have revealed a distinct mutational landscape of brain metastasis from primary tumors, regional lymph nodes and extracranial metastasis (3); however, there is a lack of evaluation regarding the magnitude of BMs from the same or different primary sites which share common mutations. In the hierarchical clustering analysis of brain metastasis samples in the present study, higher genetic similarity among BMs from lungs was not observed compared with that between BMs from lungs and other primary sites. Previous analyses have revealed independent evolution of brain metastasis from the primary sites (7,25,26). These

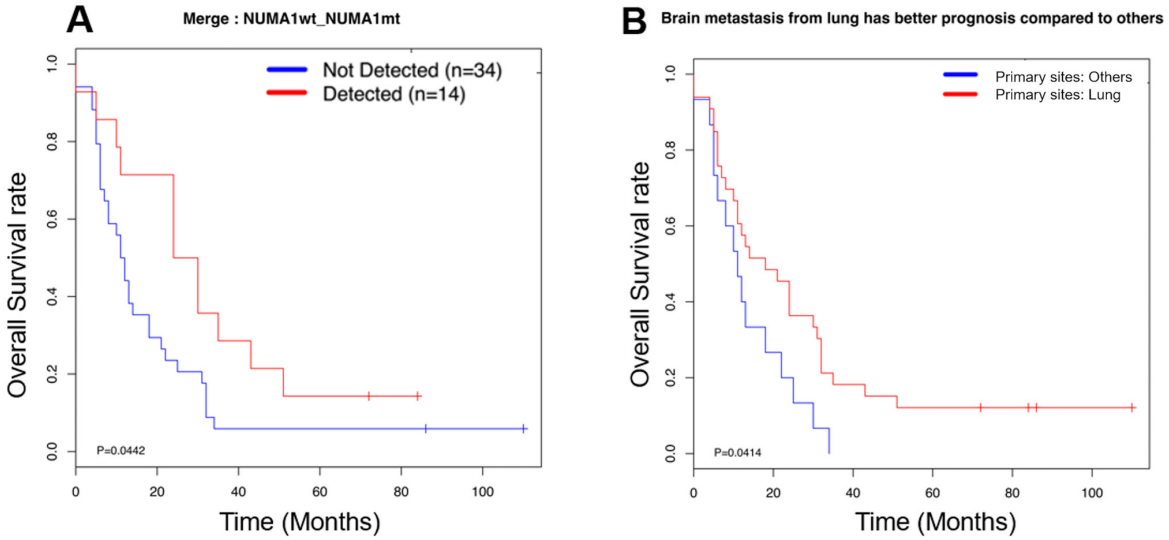

Figure 4. Overall survival rate of patients with brain metastasis in the present cohort. (A) Overall survival rate of patients with brain metastasis with (red line) or without (blue line) the *NUMA1* mutation. (B) Overall survival rate of patients with brain metastasis from the lungs (red line) and other types of cancer (blue line). *NUMA1*, nuclear mitotic apparatus protein 1.

results collectively suggested that brain metastasis should be treated by targeting genomic alterations enriched in brain metastasis instead of the primary tumors. In the present study, there were 15% of patients with BM that harbored mutations in the *PIKC3D* gene (Fig. 1), which was consistent with previous reports that PI3K could be a potential brain metastasis therapeutic target (27,28).

Comparisons of the mutational landscape of brain metastasis with that of the primary tumors revealed potential driver mutations for brain metastasis in the *KRAS*, *PI3K* and DNA damage response signaling pathways (7,23,29,30). It is largely unknown whether prognosis would be affected if patients harbor these mutations in their primary sites. Potential mutations that could contribute to brain metastasis from the lungs were identified by comparing the frequency of recurrent mutations to their frequencies in the lung cancer data from TCGA. Gene mutations associated with DNA damage response deficiency were enriched in brain metastasis samples, and patients with the *NUMA1* mutation exhibited a shorter progression-free survival time.

The tumor suppressor gene, *TP53*, has antiproliferative effects, and somatic *TP53* gene alterations are frequent in most types of human cancer (31). It also regulates the transcription of genes involved in processes that are essential for metastasis, such as cell motility and adhesion (32,33). A high mutation frequency of *TP53* was identified in BMs in the present study, and a high *TP53* mutation frequency has also been observed in samples of brain metastasis of breast cancer (34-36). Therefore, these data collectively suggested that the *TP53* mutation not only contributed to the development of tumors at the primary sites, but also promoted brain metastasis.

Identifying mutations affecting the survival of patients with brain metastasis is fundamental for developing therapeutic approaches for brain metastasis. *NUMA1* interacts and colocalizes with the P53-binding protein 1 (P53BP1), which prevents P53BP1 accumulation at the DNA break, and high *NUMA1* expression predicts improved patient outcomes (17). *NUMA1* also promotes p53-dependent downstream gene transcription in cancer cells (37,38). It was speculated that loss-of-function of NUMA1 affects the DNA damage response and may limit the expansion of brain metastasis subclones. *NUMA1* alternative splicing has been identified to be involved in multiple primary cancer sites (39), and has recently been reported to be enriched in prostate cancer brain metastasis (15). However, when comparing the survival time of patients with brain metastasis with or without each recurrent mutation, a missense mutation in the structural nuclear protein, *NUMA1*, was associated with a longer survival time compared with that of patients without this mutation. Collectively, the *NUMA1* mutation in the primary sites caused more frequent brain metastasis. However, patients with BMs and the *NUMA1* mutation had a good prognosis, suggesting that the role of the DNA damage response in the formation of brain metastasis and the clinical outcome of brain metastasis may be independent of each other.

In conclusion, BM originates from distinct sites; however, the primary tumor may have different mutational signatures, and it was found that brain metastases from different sites shared commonly mutated genes. In the patients with lung cancer and brain metastasis, recurrent mutations with a higher mutation rate in brain metastasis compared with that at the primary site were found, indicating that these genes are potential brain metastasis driver genes. Analysis of the TCGA lung cancer dataset revealed that potential brain metastasis driver genes were associated with poor progression-free survival.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

WJ, CZ and DZ conceived and designed the present study. DZ, SM, BM, XG and KS collected samples, performed the experiments and recorded the clinical information. XW, WZ, JP, PL and FX processed and analyzed the data. DZ, XW, CZ and WJ drafted the initial manuscript with input from all authors. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Beijing Tiantan Hospital, Capital Medical University (Beijing, China) and performed in accordance with the principles of the Declaration of Helsinki. Written informed consent was provided by all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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