Whole exome sequencing (WES) analysis of transformed small cell lung cancer (SCLC) from lung adenocarcinoma (LUAD)

Tongji Xie[1], Yan Li[1], Jianming Ying[1], Weijing Cai[2], Junling Li[1], Kye Young Lee[3], Biagio Ricciuti[4,5], Jose Pacheco[6], Puyuan Xing[1]

[1]National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China; [2]Shanghai Tongshu Biotechnology Co., Ltd, Shanghai, China; [3]Precision Medicine Lung Cancer Center, Konkuk University Medical Center and Department of Pulmonary Medicine, Konkuk University School of Medicine, 120-1 Neungdong-ro, Gwangjin-Gu, Seoul, 05030 Republic of Korea; [4]Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA; [5]Department of Experimental, Diagnostic and Specialty Medicine, S. Orsola-Malpighi University Hospital, Alma Mater Studiorum University of Bologna, Via Massarenti 9, 40138 Bologna, Italy; [6]University of Colorado Anschutz Cancer Center, Aurora, CO, USA

Contributions: (I) Conception and design: T Xie, P Xing; (II) Administrative support: J Li; (III) Provision of study materials or patients: P Xing, Y Li; (IV) Collection and assembly of data: J Ying; (V) Data analysis and interpretation: T Xie, W Cai; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Puyuan Xing. Department of Oncology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, No 17 Panjiayuan Nanli, Chaoyang district, Beijing 100021, China. Email: xingpuyuan@cicams.ac.cn.

Background: Histologic transformation of non-small cell lung cancer (NSCLC) to small cell lung cancer (SCLC) is a rare mechanism of acquired resistance to epidermal growth factor receptor (*EGFR*)-targeted tyrosine kinase inhibitors. However, the SCLC transformation has also been observed in non-*EGFR*-mutant NSCLC. In these cases, whether SCLC initially co-exists with NSCLC or originates from initial NSCLC remains to be determined.

Methods: Whole exome sequencing was performed on 10 samples from 5 patients with SCLC transformation from lung adenocarcinoma (LUAD), a main subtype of NSCLC. Somatic mutations and copy number variations (CNVs) were analyzed to explore the differences between initial LUAD and transformed SCLC, as well as the origin of transformed SCLC.

Results: After SCLC transformation, the mutation spectrum changed, with decreased C>T and increased C>A. Compared with initial LUAD, the CNV burden of transformed SCLC was greatly increased (39.0 *vs*. 61.1, Wilcoxon P=0.4). The higher the CNV burden of LUAD, the shorter the time to SCLC transformation was observed to be; and the higher the CNV burden of transformed SCLC, the shorter the overall survival (OS) after transformation. Clonal evolution analysis showed different clonal components between initial LUAD and transformed SCLC.

Conclusions: The transformation of LUAD into SCLC may be promoted by CNV events rather than mutational events. CNV burden was associated with the time to SCLC transformation and with the OS of patients following SCLC transformation. Transformed SCLC did not evolve directly from the initial LUAD but branched off from LUAD before the time of initial diagnosis.

Keywords: Next generation sequencing (NGS); single nucleotide variants (SNVs); copy number variation (CNV) burden; clonal evolution analysis; prognostic predictor

Introduction

Histologic transformation of *EGFR*-mutant non-small cell lung cancer (NSCLC) to small cell lung cancer (SCLC) represents a rare mechanism of acquired resistance to epidermal growth factor receptor (*EGFR*)-targeted tyrosine kinase inhibitors (TKIs), occurring in 3–14% of *EGFR*-mutant NSCLCs (1). The phenomenon of SCLC transformation in TKI-resistant *EGFR*-mutant cancers has been identified in small retrospective series and case reports (2-10). The transformation of NSCLC to SCLC has also occasionally been observed in *EGFR* wild type NSCLC (9,10), raising the question of whether SCLC initially co-exists with NSCLC or rather originates from initial NSCLC.

Several studies have been conducted to elucidate this mechanism. For instance, Niederst *et al.* (11) demonstrated that while the original *EGFR* mutation was maintained in transformed cancers, the expression of the *EGFR* protein was significantly diminished after transformation, thus rendering the transformed tumors unresponsive to *EGFR* TKI therapy. However, the mechanisms underlying low expression of *EGFR* in SCLC remain unknown. Oser *et al.* (12) suggested that, since every transformed SCLC tumor sample in their study had retained its original *EGFR*-activating mutation, transformed SCLCs are not independent de-novo cancers but a transformed phenotype, which is in contrast with the hypothesis that patients had tumors with combined histology at the time of initial diagnosis, with the SCLC component becoming dominant over time, following treatment with *EGFR* TKIs. Lee *et al.* (13) showed that the SCLC clone branched off from the founder clone early, in some cases even before initial clinical cancer diagnosis, and that NSCLCs prone to transformation may show both tumor protein p53 (*TP53*) and *RB1* inactivation at initial diagnosis. Consistently, retrospective analyses have shown that concurrent *RB1* and *TP53* alterations at baseline define a unique subset of *EGFR*-mutant NSCLCs at increased risk of SCLC transformation, and poor clinical outcomes (1,14). A recent study reported the association between SCLC transformation and the resistance to ROS TKIs in a patient with *ROS1* fusion-positive (*ROS1*+) lung cancer (15), in which WES was performed in order to define the clonal evolution of the transformed SCLC. However, the tumor tissue from the initial or ROS TKI-resistant LUAD was insufficient for WES, so the WES based clonal evolution analysis of the transformed SCLC from the preceding

LUAD was unavailable. According to the findings that while the *ROS1* fusion was retained throughout the evolutionary trajectory, its expression was lost, and the *ROS1* G2032R resistance mutation previously detected in the ROS TKI-resistant LUAD was not identified in the transformed SCLC, the authors deduced that the transformed SCLC and the preceding LUAD shared a common clonal origin and had an early divergence.

Against this background, we aimed to explore the genomic similarities and differences between initial lung adenocarcinoma (LUAD), the most common subtype of NSCLC and transformed SCLC, and to investigate the origin of transformed SCLC using clonal evolution analysis.

We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/tlcr-20-1278).

Methods

Patient samples and clinical characteristics

Five patients with transformed SCLC from NSCLC who underwent lung biopsy at the Cancer Hospital of the Chinese Academy of Medical Sciences and Peking Union Medical College between November 2013 and January 2019, were selected for this study. The study was approved by the ethics committee of the Cancer Hospital of the Chinese Academy of Medical Sciences (No. 18-102/1680). Written informed consent was obtained from all study participants. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Whole exome sequencing was performed on samples collected from each patient at the time of initial LUAD diagnosis and at the time of transformed SCLC diagnosis (Shanghai Tongshu Biotechnology Co., Ltd, Shanghai, China).

We selected previously reported patients in the Chinese LUAD cohort who did not transform to SCLC until death as controls for this study (16), and finally 16 patients were included. Whole exome sequencing has been performed on the tumor samples from these patients. The basic clinical information was listed in Table S1.

DNA extraction

We performed DNA extraction from serial thick sections cut from tumor tissue samples and control sections. The invasive tumor content was estimated by pathologists, to

ensure more than 50% of cells were tumor cells. The DNA was isolated from the FFPE using the DNeasy Blood and Tissue Kit (69504, QIAGEN, Venlo, Netherlands).

EGFR mutation analysis

EGFR mutations were analyzed based on the principle of the amplification refractory mutation system (ARMS). Briefly, resected tumor samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Extracted DNA was used for PCR with the Mx3000PtM (Stratagene, La Jolla, USA) using the *EGFR* 29 Mutations Detection Kit (Amoy Diagnostics, Xiamen, China).

Whole exome sequencing

Targeted capture pulldown and exon-wide libraries were created from native DNA using the xGen® Exome Research Panel (Integrated DNA Technologies, Inc., Skokie, Illinois, US) and TruePrep DNA Library Prep Kit V2 for Illumina (#TD501, Vazyme, Nanjing, China), and paired-end sequence data were generated using Illumina HiSeq machines with an average sequencing depth of 150× for controls and 320× for tumor tissues. The sequence data were aligned to the human reference genome (NCBI build 37) using Burrows-Wheeler Aligner (BWA), and polymerase chain reaction (PCR) duplicates were sorted and removed using sambamba.

Variant calling pipeline

Single nucleotide variants (SNVs), insertions, and deletions were detected using Strelka2 with default parameters. Variants and polymorphisms were annotated using the Ensembl Variant Effect Predictor (VEP). A minimum of 20 reads covering mutated region and 5 reads supporting the variant allele are required for somatic SNV/indel calling. In contrast, sequencing depth need to be ≥20×, and reads supporting the variant <5 at the same site in the normal control sample. Variants with MAF >1% in the ExAC, gnomAD, and esp6500 databases were filtered out as common germline variants.

Tumor mutation burden

The tumor mutation burden (TMB) of a tumor sample is calculated by the number of non-synonymous somatic mutations (single nucleotide variants and small insertions/

deletions) per mega-base in coding regions. We defined the non-synonymous somatic mutations as follows: firstly, select the mutations that appeared within the consensus coding sequence region designed by the CDS project; secondly, remove variant with variant count <3, allele frequency <5% or total depth <25; at last, filter out synonymous and splice region mutations, and retain only nonsynonymous mutations (missense, nonsense, insertions and deletions mutations).

Copy number variation analysis

Somatic copy number variations (CNVs) were analyzed using FACETS, and the resulting CNVs were used in further analysis. Recurrent CNVs were identified with GISTIC2.0. Circos were plotted using RCircos.

Intratumoral heterogeneity

Intratumoral heterogeneity (ITH) was evaluated based on two characteristics of ITH, the number of clones forming the tumor (ITH index) and clonal diversity (Shannon diversity index, SDI), according to the methods described previously (17).

Clonal evolution analysis

PyClone was used to cluster subclones inferred from SNVs. With the clustering results used as input, the optimal tree solutions were obtained with the iterative version of citup. Then, the fish plot and phylogenetic tree of each patient were constructed with timescape.

Statistical analysis

All visualizations were generated in the R statistical environment (v3.6.0) using ggpubr, maftools, pheatmap, copynumber and VennDiagram packages. T-test was applied to continuous and normal distribution data; and wilcoxon rank-sum test was applied to discrete variable or non-normal distribution data. P<0.05 was considered as statistical significance.

Data availability

The data that support the findings of this study are openly available from the Sequence Read Archive (SRA) at https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA622410.

Table 1 Clinicopathologic characteristics of patients with SCLC transformation from LUAD

Patient ID	Sex	Age	Active smoking	Initial histology		Transformed histology		Treatment before Transformation	Time (months)		
				Histology	EGFR mutation	Histology	EGFR mutation		Time of SCLC transformation	OS after transformation	Total OS
P1	Female	60	Never	LUAD	Ex19Del	SCLC	Ex19Del	Chemotherapy, gefitinib, icotinib, afatinib	42.7	20.7	63.4
P2	Female	70	Never	LUAD	Ex19Del	SCLC	Ex19Del	Icotinib, afatinib	40.9	9.7	50.6
P3	Male	48	20 yr	LUAD	Ex19Del	SCLC	Ex19Del	Chemotherapy, erlotinib	11.4	27.3	38.7
P4	Female	33	Never	LUAD	Ex18G719X	SCLC	Ex18G719X	Chemotherapy, gefitinib, afatinib, bevacizumab	16.0	8.5	24.5
P5	Male	73	Never	LUAD	Ex19Del	SCLC	Ex19Del	Gefitinib, afatinib	56.2	>14.5	Alive

SCLC, small cell lung cancer; LUAD, lung adenocarcinoma.

Our scripts are available at https://github.com/Chitanda-Satou/BioinformaticsScripts. All other data are available upon reasonable request.

Results

Clinicopathological characteristics

Five patients who were initially given a diagnosis of advanced LUAD with canonical *EGFR*-activating mutation and underwent *EGFR* TKI treatment to achieve a favorable response were selected. The clinical characteristics of these patients are summarized in *Table 1*. Two patients (patients 1 and 3) were excluded from further analysis. For patient 1, the quality of the WES data was poor due to the degradation of adenocarcinoma DNA; for patient 3, normal tissue was unavailable because of the patient's death. The clinical history of the three patients included in this study is presented in *Figure 1*. *EGFR* mutations were determined through amplification refractory mutation system-polymerase chain reaction (ARMS-PCR). All patients harbored *EGFR* mutations at the time of LUAD diagnosis and maintained the same *EGFR* mutation at the time of SCLC diagnosis. For patients 2, 4, and 5, the time to SCLC transformation was 40.9, 16.0, and 56.2 months, respectively, and the overall survival (OS) after transformation was 9.7, 8.5, and >14.5 months, respectively (*Table 1*).

Mutational landscape of initial LUAD and transformed SCLC

Tumor samples obtained at the time of initial LUAD diagnosis and at the time of transformed SCLC diagnosis

for each patient were subjected to WES with an average sequencing depth of 320×. Matched DNA from normal lung tissue was used as germline DNA control. The number of nonsynonymous somatic mutations per sample ranged from 106 to 468. The distribution of substitutions showed a strong preference for C>T transitions that is consistent with cytidine deamination.

After SCLC transformation, the mutation spectrum changed, with a decrease in the median proportion of C>T, from 55.8% to 33.3%, and an increase in the median proportion of C>A, from 12.7% to 25.0% (*Figure 2A*). The mutation spectrum of LUAD with or without SCLC transformation were different (Figure S1A). After SCLC transformation, TMB in patient 2 decreased slightly, but TMB in patient 4 and 5 remained unchanged. The TMB of LUAD and SCLC was 9.7 and 6.1 in patient 2, 1.5 and 1.7 in patient 4, and 1.3 and 1.7 mutations per Mb in patient 5, respectively (*Figure 2B*). The median TMB of LUAD with SCLC transformation (1.5 mutations/Mb) was comparable to LUAD without SCLC transformation (1.3 mutations/Mb, Table S2). Eleven known lung cancer driver genes (18) were detected in patients with SCLC transformation, among which *TP53* and *EGFR* had a high mutation frequency (*Figure 2C*). Fifteen known lung cancer driver genes were detected in LUAD without SCLC transformation (Figure S1B). Only two driver genes, *TP53* and *EGFR*, were shared by LUAD with or without SCLC transformation.

Next, we investigated the most frequently mutated genes in this cohort. As shown in Figure S2, no specific mutations were observed in the transformed SCLC samples. Examination of the alterations in key pathways of typical LUAD (19) (Figure S3A) and de novo SCLC (20) (Figure
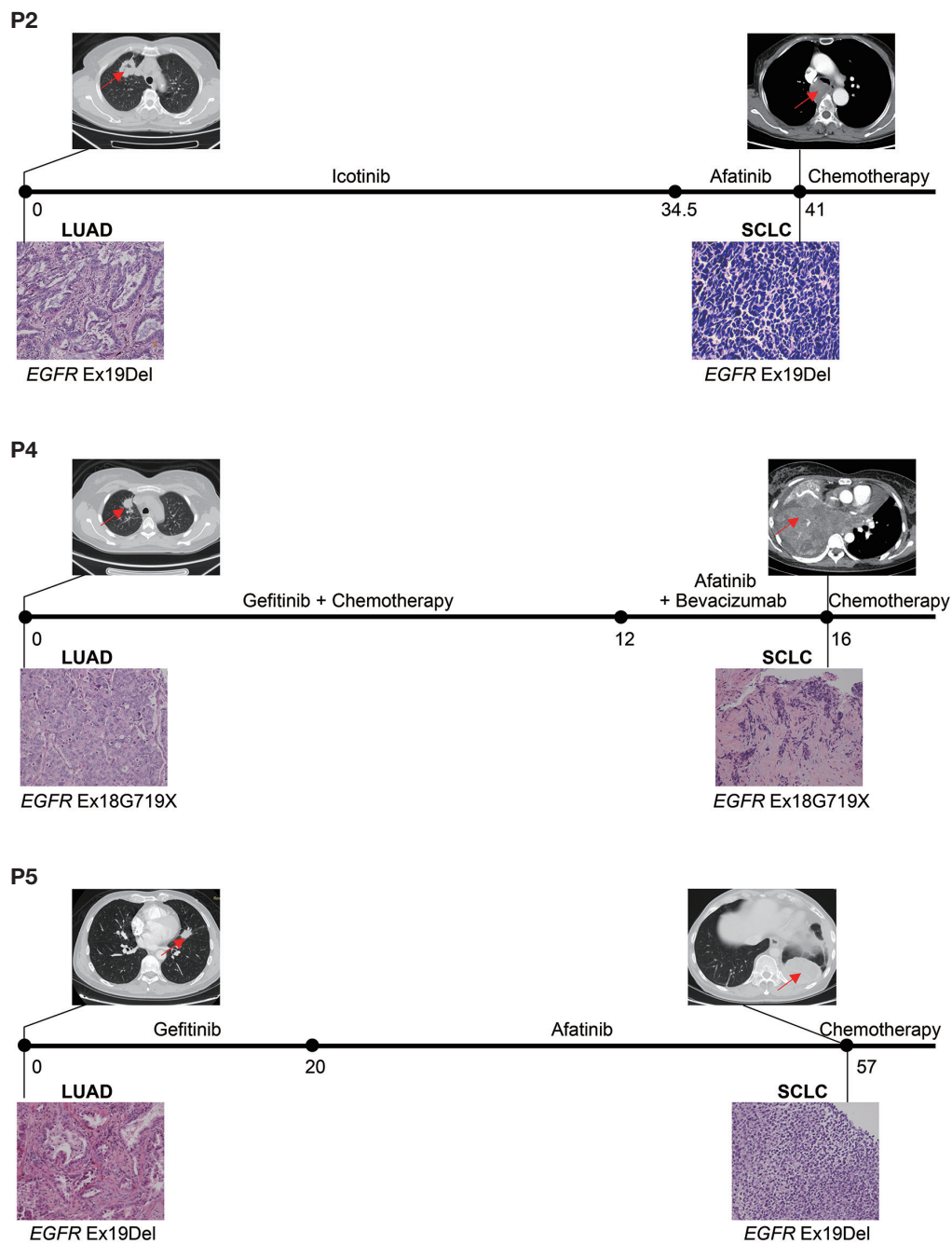
Figure 1 Clinical history of three patients with transformed small cell lung cancers (SCLCs). The staining method is hematoxylin-eosin (H&E) staining and the magnification is ×400. The timelines show the points of lung adenocarcinoma (LUAD) and SCLC tissue acquisitions, and the treatment history of the patients. Numbers indicate time (in months) from the diagnosis of LUAD. The arrows in the computed tomography images indicate LUAD or SCLC masses. EGFR, epidermal growth factor receptor.
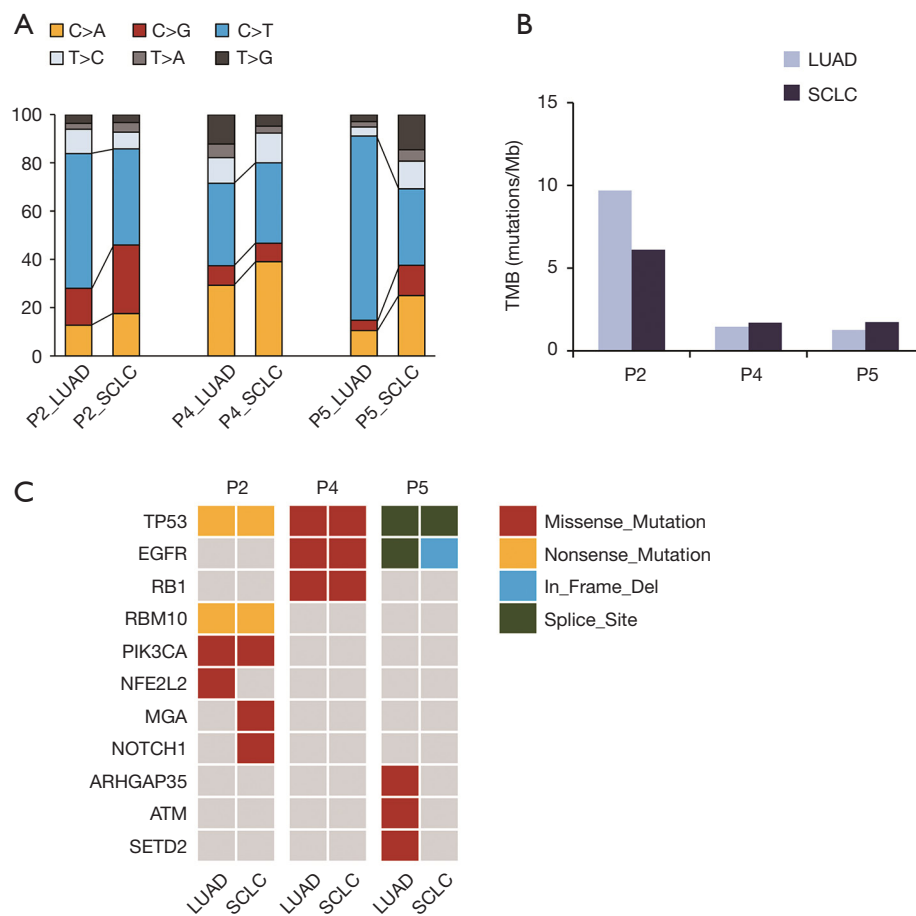
Figure 2 Mutational landscape of initial lung adenocarcinoma (LUAD) and transformed small cell lung cancer (SCLC). (A) The mutation spectrum changed after SCLC transformation. (B) The tumor mutation burden (TMB). (C) Altered known lung cancer driver genes in each sample. Each row represents a gene, each column represents a sample, and colors represent types of mutations.

S3B) revealed that the genes involved in these pathways were rarely mutated except for several recurrently mutated genes (including *TP53* and *EGFR*); however, CNVs were ubiquitous in these genes.

CNV profiles of initial LUAD and transformed SCLC

Compared with that of initial LUAD, the CNV burden of transformed SCLC was significantly elevated in patient 2 (39.0 *vs.* 94.9) and patient 5 (14.0 *vs.* 61.1), but it was slightly decreased in patient 4 (44.1 *vs.* 27.6) (*Figure 3A*). The median CNV burden of transformed SCLC was higher than that of initial LUAD (39.0 *vs.* 61.1, Wilcoxon P=0.4). However, the weighted genome instability index (wGII) (21) was comparable between initial LUAD and transformed SCLC (*Figure 3B*). As shown in *Figure 3C*, in patients 2 and

5, CNVs were widespread throughout the entire genome of transformed SCLC compared with the initial LUAD, but fewer CNVs were observed in patient 4. Known lung cancer driver genes affected by CNVs are presented in Figure S4. Compared with LUAD without SCLC transformation, the median CNV burden of initial LUAD with SCLC transformation was higher (39.0 *vs.* 21.5, Wilcoxon P=0.36, Table S2).

Clonal evolution analysis

Initial LUAD and transformed SCLC were found to share a small proportion of nonsynonymous mutations, and there were over 60% unique mutations (Figure S5). Four genes, *TP53*, mucin-17 (*MUC17*), EGFR, and pumilio RNA binding family member 1 (*PUM1*), were frequently mutated

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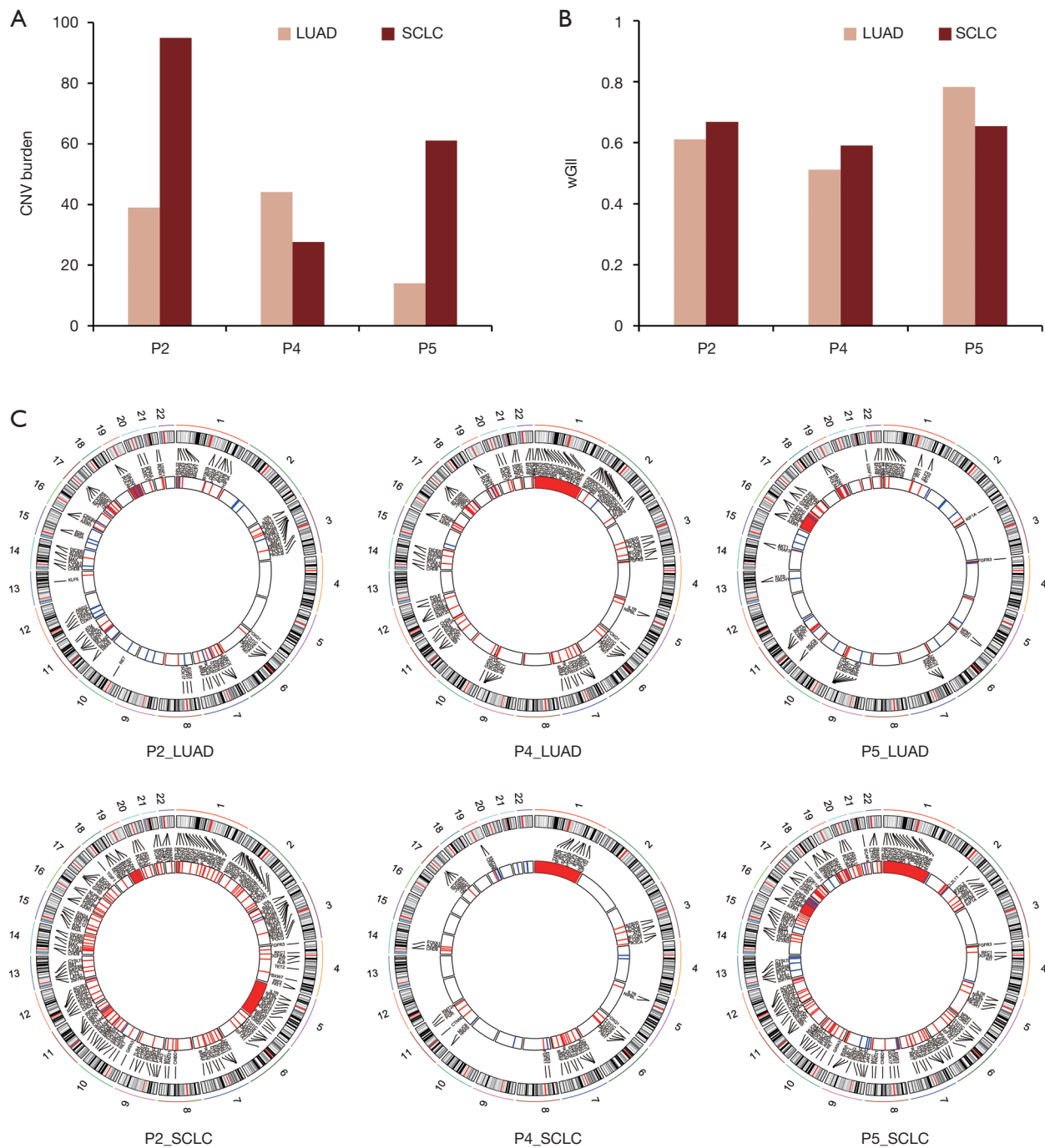
Figure 3 Copy number variant (CNV) profiles showed significant alterations in transformed SCLC. The CNV burden (A) and the weighted genome instability index (wGII) (B) of each patient. (C) Circos plot represents the genome-wide distribution density of each sample (in clockwise order). The outer ring of the plot shows the chromosomes. The central ring shows the driver genes located in the altered regions. The inner ring exhibits the CNV amplifications (red) and deletions (blue). LUAD, lung adenocarcinoma; SCLC, small cell lung cancer.
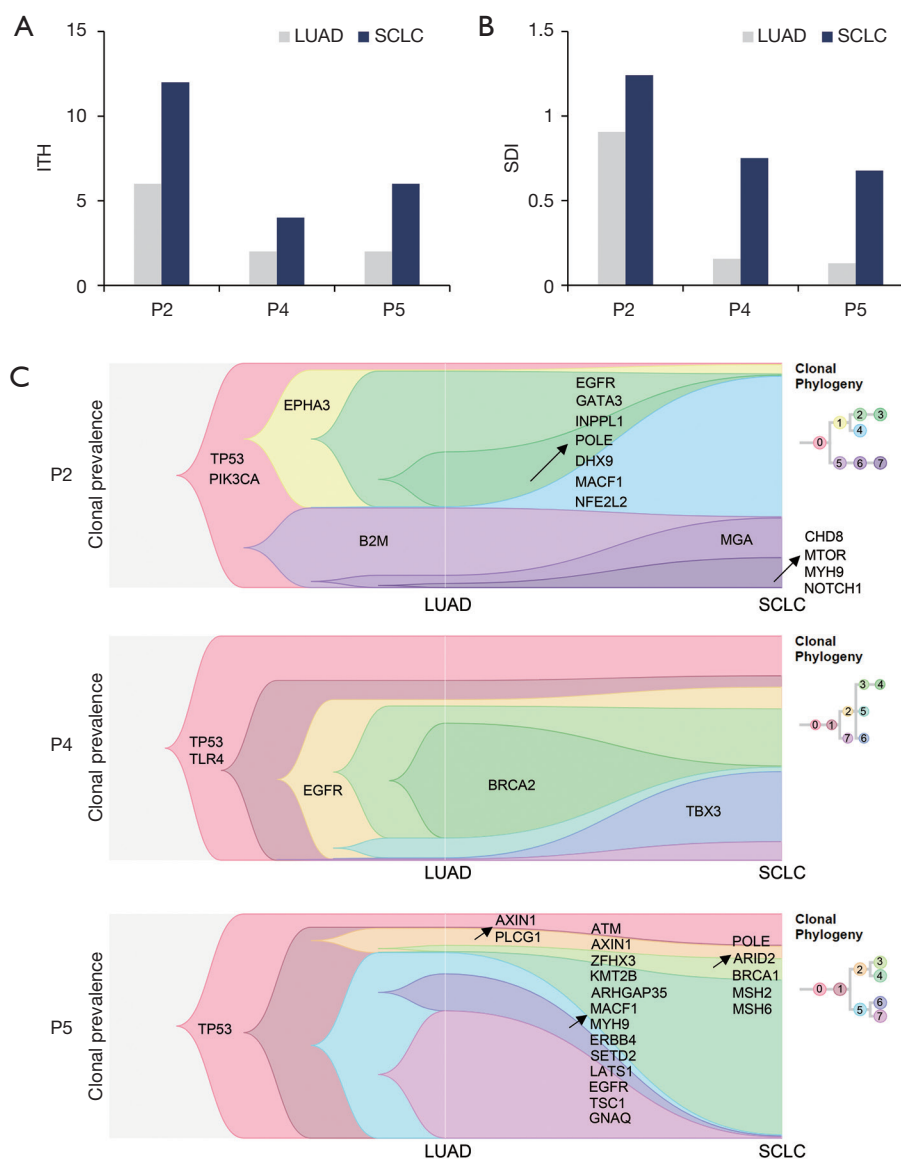
Figure 4 Clonal evolution analysis. The intratumoral heterogeneity (ITH) index (A) and Shannon diversity index (SDI) (B) of transformed SCLC was higher than that of initial LUAD. (C) Fish plots constructed by timescape. Colors indicate different clones. Driver genes detected in the clones are shown. LUAD, lung adenocarcinoma; SCLC, small cell lung cancer.

in both initial LUAD and transformed SCLC (Figure S2). The intratumoral heterogeneity (ITH) index (*Figure 4A*) and Shannon diversity index (SDI) (*Figure 4B*) of transformed SCLC were higher than those of initial LUAD. Histograms of CCF distribution also showed transformed SCLC to have a higher ITH index (Figure S6).

The clonal evolution analysis showed that the clonal components of initial LUAD and transformed SCLC were different. In patient 2, the LUAD was mainly composed of clones 2, 3, and 5, while the SCLC was mainly composed of clones 4, 6, and 7 (*Figure 4C*). The LUAD of patient 4 was mainly composed of clones 0 and 4, while the SCLC was mainly composed of clones 0, 3, and 6 (*Figure 4C*). In patient 5, the LUAD was mainly composed of clones 6 and 7, and the SCLC was mainly composed of clones 0 and 4 (*Figure 4C*). As shown, the major clones in the transformed SCLC did not originate from the major clones in the LUAD; rather, they originated from subclones generated

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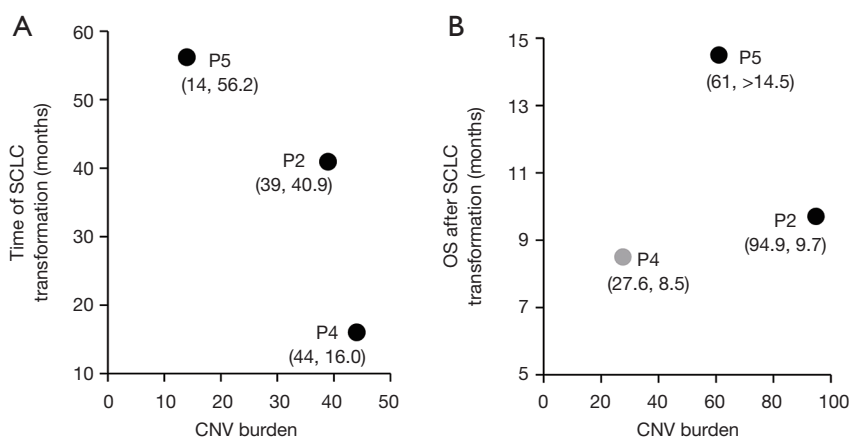
Figure 5 Copy number variant (CNV) burden was associated with the prognosis of patients with transformed SCLC. (A) The association between CNV burden and the time to SCLC transformation from LUAD. (B) The association between CNV burden and OS after transformation to SCLC. LUAD, lung adenocarcinoma; SCLC, small cell lung cancer.

before diagnosis. In all the three cases, mutations in TP53 occurred early in the tumorigenesis, and mutations in *EGFR* occurred relatively late. Other driver genes detected in the clones varied between the patients. These results suggest that LUAD and transformed SCLC originate from the same clone but the transformed SCLC clones do not originate directly from the major clones of the initial LUAD.

CNV burden is associated with the prognosis of patients with transformed SCLC

For the three patients (patient 5, 2, 4) in this study, the transformation time from LUAD to SCLC was 56.2, 40.9, and 16 months, and the CNV burden of LUAD was 14.0, 39.0, and 44.1, respectively (*Table 1*, *Figure 5A*). Together with the result that showed higher CNV burden of initial LUAD with SCLC transformation than LUAD without SCLC transformation (Table S2), these results suggested that the higher the CNV burden, the shorter the time to transformation into SCLC. The OS of the patient 5, 2 and 4 after SCLC transformation was >14.5, 9.7, and 8.5 months, with a CNV burden of SCLC of 61, 94.9, and 27.6, respectively (*Table 1*, *Figure 5B*). Because of the small sample size (N=3) whether CNV burden of transformed SCLC is prognostic remains to be determined.

Discussion

Transformation from LUAD into SCLC is a rare event in patients with lung cancer, and occurs in approximately

3–7% of *EGFR*-mutant NSCLC treated with TKIs. In this study we investigated the genomic correlates of SCLC transformation from 3 cases of *EGFR*-mutant LUAD. Although limited by the small sample size, our analysis provided some important insights into the genetic characteristics of transformed SCLC from initial LUAD.

C>T transitions were predominant in all three patients included in our analysis, all of whom were never-smokers. This finding is consistent with a previous study in which C>T transitions were found to be predominant in never-smokers and former light smokers with lung cancer, whereas a predominance of C>A transversions was observed in smokers (22). C>T transitions are resulted from the deamination of cytosine to uracil. A recent study suggested that activation-induced cytidine deaminase hypermutation could serve as an early predictor of SCLC transformation (14). In our study, C>T transitions were decreased in transformed SCLC, while C>A transversions were increased. Cancers with enrichment of C>T transitions have been reported to exhibit low levels of chromosomal alterations, and cancers with a preponderance of C>A transversions have been observed to have significantly more copy number gains (23), which is consistent with our findings. We also observed a significantly higher CNV burden in transformed SCLC than in initial LUAD in patients with increased C>A transversions and decreased C>T transitions. However, compared with that of initial LUAD, the wGII of transformed SCLC did not change, which allowed us to conclude that genome instability was not the cause of the increase in CNVs. Moreover, patients 2 and 5 had

more DDR gene alterations in their transformed SCLC than patient 4 whose CNV burden did not increase after transformation to SCLC, which indicates that incorrect repair of DNA damage may be the source of increased CNVs (24). It is not clear whether the hypothetical mechanism of SCLC transformation for non-smokers is applicable to smokers.

The origin of SCLC subclones is a question still in need of investigation. Several studies have suggested that *EGFR*-mutant lung cancers that develop in alveolar type II cells might have the potential to transform into SCLC (25-27). Lee *et al.* suggested that *EGFR* TKI-resistant SCLCs may branch out early from the LUAD clones, even before *EGFR* TKI treatment (13). Our results were consistent with the findings of Lee *et al.*, suggesting that SCLC diverges early from LUAD. We also found TP53 mutation to be an early event in both LUAD and SCLC, while *EGFR* mutation occurred later.

In previous studies, deactivation of *TP53* and *RB1* has been reported to occur in the majority of transformed SCLCs (13). In our study, all three cases harbored *TP53* mutations in both LUAD and SCLC, and one of the cases harbored *RB1* mutations. Moreover, CNV loss of *RB1* occurred in all but one of six samples. However, accumulating experimental evidence has demonstrated that while dual inactivation of RB and p53 is essential for SCLC lineage transformation in *EGFR*-mutant LUAD, it is not sufficient to induce this transformation alone, which suggests that additional factors are required (11). A retrospective study described the clinical outcomes of patients with *EGFR*-mutant transformed SCLCs. Among 67 patients, all maintained their founder *EGFR* mutation, and 15 of 19 with prior *EGFR* T790M positivity were T790 wild-type at transformation. Other recurrent mutations identified in SCLC samples were *TP53* (79%, 38/48), RB1 (58%, 18/31), and Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA*) (27%, 14/52) (1). *MYC* amplification and *PIK3CA* mutation have been proposed to potentially cooperate with RB/p53 loss to facilitate transformation, and specific epigenetic regulators may also provide the appropriate context for lineage reprogramming to occur (13). Park *et al.* (28) reported that loss of RB and inactivation of p53 combined with expression of myristoylated *AKT1* and overexpression of *MYC* and B-cell lymphoma 2 (*BCL2*), leads to the development of aggressive SCLC. In our study, in transformed SCLCs, 100% [6/6] of samples had *TP53* mutations, 33.3% [2/6] of samples had *RB1* mutations, and 33.3% [3/6] of samples had *PIK3CA* mutations. We attempted to explore the genetic features of transformed SCLC, but no common mutations were found among the 3 cases. Our failure to determine the genetic features of transformed SCLC may mainly be attributed to the number of patients in this study, which was too small to identify common features.

The results of previous studies indicate that transformed SCLC takes on many features of de novo SCLC, including inactivation of the tumor suppressors RB and p53 in nearly all cases, similar gene expression profiles, including reduced or absent *EGFR* expression, and heightened sensitivity to BCL-2 family inhibition (11,29). However, a systematic review showed that current treatment strategies derived from de novo SCLC seemed to be largely inefficacious for transformed SCLC (30). Another previous study showed that few de novo SCLCs harbored *EGFR* mutations (12). From these observations, transformed SCLC may be genetically different from de novo SCLC.

Furthermore, we found that the CNV burden of initial LUAD with SCLC transformation was higher than LUAD without SCLC transformation. There was an association between CNV burden and the prognosis of patients with transformed SCLC. We observed that the higher the CNV burden in the initial LUAD, the shorter the transformation time to SCLC, and the higher CNV burden in the transformed SCLC, the shorter the OS after SCLC transformation. Nevertheless, the value of CNV burden in predicting SCLC transformation has not been studied. In future studies, we will employ transcriptome and epigenome analysis to elucidate the causal relations between increased CNV burden and SCLC transformation.

In conclusion, we demonstrated that the transformed SCLC did not evolve directly from the initial LUAD but branched off from LUAD early. The CNV burden was associated with the time to SCLC transformation and with the OS of patients after SCLC transformation.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the MDAR

reporting checklist. Available at http://dx.doi.org/10.21037/tlcr-20-1278

Data Sharing Statement: Available at http://dx.doi.org/10.21037/tlcr-20-1278

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tlcr-20-1278). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the ethics committee of the Cancer Hospital of the Chinese Academy of Medical Sciences (No. 18-102/1680). Written informed consent was provided by all participants. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

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