Comprehensive genomic profiling of combined small cell lung cancer

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Background: Combined small cell lung cancer (CSCLC) is an uncommon and heterogeneous subtype of small cell lung cancer (SCLC). However, there is limited data concerning the different molecular changes and clinical features in CSCLC compared to pure SCLC.

Methods: The clinical and pathological characteristics of pure SCLC and CSCLC patients were analyzed. Immunohistochemistry and microdissection were performed to isolate the CSCLC components. Further molecular analysis was carried out by next-generation sequencing (NGS) in 12 CSCLC and 30 pure SCLC.

Results: There were no significant differences in clinical features between CSCLC and pure SCLC. Overall survival (OS) of CSCLC patients was worse than pure SCLC (P=0.005). NGS results indicated that TP53 and RB1 were the most frequently mutated genes in both CSCLC (83.33% and 66.67%) and pure SCLC (80.00% and 63.33%) groups. However, less than 10% common mutations were found in both CSCLC and pure SCLC. When analyzing the data of SCLC and non-small cell lung cancer (NSCLC) components of CSCLC, more than 50% common mutations, and identical genes with mutations were detected. Moreover, there were also common biological processes and signaling pathways identified in CSCLC and pure SCLC, in addition to SCLC and NSCLC components.

Conclusions: There were no significant differences in terms of clinical features between CSCLC and pure SCLC. However, the prognosis for CSCLC was worse than pure SCLC. NGS analysis suggested that
Introduction

Lung cancer is the leading cause of cancer mortality worldwide and has the highest morbidity among all cancers (1). Overall, approximately 15% of lung cancer cases are diagnosed as small cell lung cancer (SCLC) (1,2). Combined small cell lung cancer (CSCLC), a rare subtype of SCLC, consists of a heterogeneous neoplasm with SCLC plus a non-small cell cancer (NSCLC) component. In this setting, the NSCLC component may manifest as adenocarcinoma (AD), squamous cell carcinoma (SCC), large cell neuroendocrine carcinoma (LCNC), or sarcomatoid lung cancer (3-5). To date, despite standard chemotherapy and radiation regimens, the survival of SCLC is poor (6). As for CSCLC, few studies have been conducted to characterize the features of this rare subtype.

It is well acknowledged that heavy smoking contributes greatly to the development of SCLC, however, 2–3% of SCLC patients are reported to be never smokers (7,8). Molecular studies have demonstrated that genomic aberrations, including tumor protein P53 (TP53), retinoblastoma 1 (RB1) mutations, and Myc proto-oncogene protein (c-Myc) amplification, are associated with the tumorigenesis and aggressive features of SCLC (9-11). Moreover, SCLC has been shown to be associated with a higher tumor mutational burden (TMB), which may favor the use of the chemo-immunotherapy as first-line treatment (IMPOWER-133 and CASPIAN trials) or immunotherapy in treatment for further lines (12-14). In addition, immunotherapy seems to be more effective in patients with SCLC who have primary resistance to platinum, a phenomenon that is associated with the presence of mutations in DNA damage response (DDR) and DNAH10 with a higher TMB (15-17). However, owing to the lower rate of CSCLC and the rarity of resected samples, the exact genomic landscape of this entity remains unknown.

Emerging evidence shows that NSCLC originates from either basal cells in the bronchial membrane or bronchoalveolar junction, whereas SCLC generally arises from neuroendocrine cells underneath the basal bronchial membrane and alveolar type 2 (AT2) cells of the most distal region of the lung (18-20). Diverse studies of in vitro and murine models aimed at exploring the origin of the transformation of epidermal growth factor receptor (EGFR) positive ADs in SCLC after tyrosine kinase exposure have evaluated the role of type II pneumocytes (21). Sutherland et al. previously reported that targeted disruption of Tp53 and Rb1 in AT2 cells led to the development of SCLC, albeit at a much lower frequency than that of neuroendocrine cells (19). Nonetheless, the cellular origin of CSCLC remains unclear. Some reports have suggested that the components of CSCLC derive from a single pluripotent clone before random genetic aberrations occur, whereas other evidence indicate that the components of CSCLC may co-exist in a single tumor (22,23).

Due to the paucity of reports, this current study aimed to explore the clinical, pathological, and molecular differences between CSCLC and pure SCLC and the prognosis of these tumor subtypes. To explore the possible origin of the different components of CSCLC, we applied a next-generation sequencing (NGS) approach for comprehensive genomic profiling.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/tlcr-20-1099).

Methods

Patients

Patient data from a total of 324 patients with SCLC resected between January 2014 to July 2019 were obtained from Shanghai Pulmonary Hospital. Clinical data about gender, age, smoking history, pathological characteristics, postoperative treatment etc. were collected from the medical records. Telephone follow-up and outpatient records were
used for survival data, and the cutoff date for follow-up of the current study was September 30, 2019. Pathological features of all samples were revised and confirmed independently by two certified pathologists (L. Zhang and L. Hou). The pathological stage was determined based on the eighth edition of the American Joint Committee on Cancer tumor-node-metastasis (TNM) staging system for lung cancer (24). This study was approved by the Institutional Review Board of Shanghai Pulmonary Hospital (ethical approval number: K18-066). Moreover, all procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was taken from all the patients.

Immunohistochemistry (IHC)

First, 4-µm slices were dyed with hematoxylin and eosin (HE), and the histological type was analyzed by selecting different markers using IHC. After dewaxing in xylene and rehydrating in gradient ethanol, the slides were prepared for antigen retrieval by boiling them in 10-mM sodium citrate buffer. Then, 3% hydrogen peroxide was used to block the endogenous peroxidase activity of the slides. Thereafter, the samples were incubated with the following primary antibodies at 4 ℃ overnight using thyroid transcription factor 1 (TTF-1) (clone 8G7G3/1, DAKO; dilution 1:200), napsin A (polyclone, Abcam; dilution 1:250), P40 (polyclone, Abcam; dilution 1:200), cluster of differentiation 56 (CD56, clone 123C3, DAKO; dilution 1:100), synaptophysin (Syn, clone DAK-SYNAP, DAKO; dilution 1:50), cytokeratin 5/6 (CK5/6, clone D5/16 B4, DAKO; dilution 1:100), and chromogranin A (CgA, clone DAK-A3; dilution 1:200). The slides were subsequently incubated with an anti-primary antibody and then stained with diaminobenzidine and hematoxylin. Finally, they were dehydrated in ethanol, washed in xylene, and then covered with coverslips for further microscopic observation.

Sample collection and preparation

The formalin fixed paraffin-embedded (FFPE) samples were obtained from patients who underwent surgery, and detailed information were reported in Tables S1,S2. After staining with HE and with different markers of IHC, the morphologies of all of the samples were confirmed by two senior pathologists, independently. The CSCLC cases with a clear morphological boundary of disparate contents were selected for microdissection and further subjected to genomic profiling performed in the OrigiMed laboratory (Yuansu, OrigiMed Inc., China).

Targeted NGS and genetic analysis

NGS was performed as previously described (16,25). Briefly, FFPE tumor tissues were collected for the detection of genetic alterations. Yuansu 466 panel covered all the coding exons of the 466 cancer-related genes and selected introns of 36 genes that were frequently rearranged in solid tumors (table online: https://cdn.amegroups.cn/static/public/tlcr-20-1099-3.docx). Then, 50–250 ng of DNA was extracted, and libraries were constructed and sequenced on an Illumina NextSeq 500 (San Diego, CA, USA) with a mean coverage of at least 700×. Genomic alterations consisting of single-nucleotide variations (SNV), copy-number variations (CNV), gene fusions/ rearrangements, and short or long insertions/deletions (indels) were identified for advanced analysis. All genomic alterations for the examined tissues in the current study are summarized in https://cdn.amegroups.cn/static/public/tlcr-20-1099-3.docx. Normally, identical genes with the same DNA change indicated a common mutation (26). And we also defined identical genes with a different variant type as identical genes with mutations in current study (Figures S1,S2).

Statistical analysis

SPSS 21.0 software (SPSS Inc., USA) was conducted for statistical analysis. The significances of the parameters between CSCLC and pure SCLC were determined by the χ² test or Fisher’s exact test, as appropriate. The survival curves were calculated using the Kaplan-Meier method, and significance was analyzed by the log-rank test. A P value <0.05 was defined as statistically significant.

Results

Clinical characteristics and survival

Among all patients with resected SCLC, 52 patients (16%) were diagnosed as CSCLC, while the remaining 272 patients were confirmed as pure SCLC. The median age for all SCLC patients was 63 years (range, 31 to 83 years), while for CSCLC and pure SCLC the median age was 65 years (range, 31 to 78 years) and 63 years (range, 31 to 83 years), respectively. Upon comparing clinico-
pathological characteristics between the CSCLC and SCLC groups, no significant difference was revealed (Table 1). The median time for follow-up was 17 months (range, 2 to 66 months). We found that the median overall survival (OS) for pure SCLC and CSCLC was 60 and 22.9 months, respectively. Additionally, the OS of pure SCLC (95% CI: 42.79 to 51.07) was better in respect to CSCLC (95% CI: 27.00 to 47.47) as shown in Figure 1.

**IHC characteristics**

Among the 12 cases of CSCLC, SCC components were discovered in 6 cases, while AD components were identified in the remaining 6 cases (Figure 2). Neuroendocrine markers (CD56, Syn, and CgA) were positive-stained in 83.33% [10/12], 83.33% [10/12], and 41.67% [5/12] of the SCLC elements, respectively. P40 staining was positive in the SCC components [5/6] and negative in the SCLC components. TTF-1 staining was positive in 83.33% [5/6] of the AD components and 58.33% [7/12] of the SCLC components. Napsin A was expressed positively in 50.00% [3/6] of the AD components and negatively in the SCLC components (Table S3).

**The mutational landscape in CSCLC and pure SCLC**

To illustrate the mutational landscape in CSCLC and pure SCLC, a targeted NGS panel was adopted. In all, 12 cases diagnosed as CSCLC and 30 cases diagnosed as pure SCLC were successfully dissected, and the DNA from each sample was extracted, qualified, and sequenced. This yielded 267 mutations within 192 genes in pure SCLC cases, and 385 mutations within 200 genes in pure CSCLC samples (Figure 3, online material: https://cdn.amegroups.cn/static/public/tlcr-20-1099-1.docx and https://cdn.amegroups.cn/static/public/tlcr-20-1099-2.docx). To determine the possible role of the mutated genes in CSCLC and pure SCLC, we further analyzed the biological processes and pathways involved using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) in The Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/summary.jsp). We found that the mutated genes might be involved in the following common biological processes: GO:00018108 (peptidyl-tyrosine phosphorylation), GO:0048015 (phosphatidylinositol-mediated signaling), GO:0046777 (protein autophosphorylation), GO:0007169 (transmembrane receptor protein tyrosine kinase signaling pathway), GO:0045944 (positive regulation of transcription from RNA polymerase II promoter), GO:0014066 (regulation of phosphatidylinositol 3-kinase signaling), GO:0045893 (positive regulation of transcription, DNATemplated), GO:0002828 (positive regulation of cell proliferation), GO:0007165 (signal transduction), and GO:0007050 (cell cycle arrest) (Figure 4A,B). Moreover, the mutated genes in the two groups of tumors have been implicated in some common signaling pathways, such as Wnt, p53, and notch signaling pathways (Figure 4C,D).

Further analysis found that 22 common mutations and 104 identical genes with mutations were determined in CSCLC and pure SCLC, occupying 8.24% and 54.17% of CSCLC and 5.71% and 52.00% of pure SCLC, respectively (Figures S1A,S2A). Among the top 10 genes with mutations in CSCLC and pure SCLC, 9 genes in CSCLC [TP53, RB1, SRY-box transcription factor 2 (SOX2), EGRF, telomerase reverse transcriptase (TERT), notch receptor 3 (NOTCH3), histone-lysine N-methyltransferase 2D (KMT2D), FAT tumor suppressor homolog 1 (FAT1)], and family with sequence similarity 135 member B (FAM135B) and 9 genes in pure SCLC (TP53, RB1, spectrin alpha, erythrocytic 1 (SPTA1), low-density lipoprotein receptor-related protein 1B (LRP1B), TERT, NK2 homeobox 1 (NKX2-1), NOTCH1, FAM135B, and FAT4] appeared in the abovementioned 104 genes (Figure S3). Additionally, the amplification of SOX2, MYC, AKT serine/threonine kinase 2 (AKT2), RAD54 homolog B (RAD54B), protein kinase C iota (PRKCI), cyclin D1 (CCND1), cyclin E1 (CCNE1) and fibroblast growth factors 4/3/12 (FGF4, FGF3, FGF12) were found only in CSCLC, while amplification of TERT, NKX2-1, succinate dehydrogenase complex flavoprotein subunit A (SDHA), and FGF10 could be detected in both CSCLC and pure SCLC (Figure S3).

**The mutational landscape in NSCLC and SCLC components of CSCLC**

To further investigate the correlation between NSCLC and SCLC components of CSCLC, the mutated genes of the unique component of CSCLC were analyzed. We observed 211 mutations linked to 159 genes in the SCLC component of CSCLC and 169 mutations linked to 125 genes in the NSCLC component of CSCLC (online material: https://cdn.amegroups.cn/static/public/tlcr-20-1099-1.docx and https://cdn.amegroups.cn/static/public/tlcr-20-1099-2.docx). GO analysis in DAVID showed that most of the biological processes associated with the
Table 1 Clinical and pathological parameters of CSCLC and pure SCLC

| Parameters          | Category          | Combined SCLC | Pure SCLC | $\chi^2$ | P     |
|---------------------|-------------------|---------------|-----------|----------|-------|
| Gender              | Male              | 46            | 230       | 0.5269   | 0.4679|
|                     | Female            | 6             | 42        |          |       |
| Age                 | >60               | 36            | 160       | 1.9784   | 0.1596|
|                     | ≤60               | 16            | 112       |          |       |
| Smoking             | ≥400              | 19            | 97        | 0.0146   | 0.9038|
|                     | <400              | 33            | 175       |          |       |
| Surgery type        | Limited resection | 5             | 15        | 4.3073   | 0.1161|
|                     | Lobectomy         | 46            | 231       |          |       |
|                     | Pneumonectomy     | 1             | 26        |          |       |
| Tumor site          | Left              | 26            | 147       | 0.2869   | 0.5922|
|                     | Right             | 26            | 125       |          |       |
| Pleural invasion    | Yes               | 4             | 12        | 0.4240   | 0.5150|
|                     | No                | 48            | 260       |          |       |
| Nerve invasion      | Yes               | 4             | 5         | 3.5840   | 0.0583|
|                     | No                | 48            | 267       |          |       |
| Vessel invasion     | Yes               | 9             | 31        | 1.4093   | 0.2351|
|                     | No                | 43            | 241       |          |       |
| T                   | 1                 | 18            | 121       | 3.6781   | 0.2887|
|                     | 2                 | 28            | 108       |          |       |
|                     | 3                 | 4             | 33        |          |       |
|                     | 4                 | 2             | 10        |          |       |
| N                   | 0                 | 27            | 134       | 1.9829   | 0.5733|
|                     | 1                 | 11            | 44        |          |       |
|                     | 2                 | 14            | 93        |          |       |
|                     | 3                 | 0             | 1         |          |       |
| TNM                 | I                 | 23            | 108       | 0.9455   | 0.8108|
|                     | II                | 11            | 51        |          |       |
|                     | III               | 18            | 109       |          |       |
|                     | IV                | 0             | 4         |          |       |
| Postoperative adjuvant chemotherapy | Yes | 34 | 189 | 0.3421 | 0.5586 |
|                     | No                | 18            | 83        |          |       |
| Postoperative adjuvant radiotherapy | Yes | 11 | 75 | 0.9227 | 0.3368 |
|                     | No                | 41            | 197       |          |       |
| NSE                 | Normal            | 43            | 187       | 2.9473   | 0.0860|
|                     | Abnormal          | 4             | 48        |          |       |
| CEA                 | Normal            | 40            | 217       | 2.5369   | 0.1112|
|                     | Abnormal          | 7             | 18        |          |       |

CSCLC, combined small cell lung cancer; SCLC, small cell lung cancer; NSE, neuron-specific enolase; CEA, carcinoembryonic antigen.
Mutated genes of both components of CSCLC were similar (Figure 5A,B). Additionally, the mutated genes of the two CSCLC components were both implicated in classical signaling pathways, including PI3K-Akt, p53, notch, and others (Figure 5C,D). When comparing the common mutations and mutated genes in the two components, we discovered 112 common mutations and 92 identical genes with mutations in both components. Also, the proportion of common mutations and identical genes with mutations was 53.08% and 57.86% for the SCLC component, respectively, and 66.27% and

**Figure 1** The overall survival of pure small cell lung cancer (SCLC) was better than combined small cell lung cancer (CSCLC) (P=0.005).

**Figure 2** Representative images of specific markers and the boundary of the different components of combined small cell lung cancer (CSCLC). The left panel shows the staining of small cell lung cancer (SCLC) and adenocarcinoma (AD), while the right panel shows the staining of SCLC and squamous cell carcinoma (SCC).
73.60% for the NSCLC component, respectively (Figures S1B, S2B). In terms of the common mutations in both components, 10 TP53 common mutations, followed by RB1 [7], phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA, 3), and EGFR [2] common mutations, were found in 12 samples of SCLC and NSCLC components of CSCLC (Table S4). Meanwhile, among the top 10 mutated genes of SCLC and NSCLC components of CSCLC, 10 mutated genes in SCLC [TP53, RB1, SOX2, PIK3CA, NOTCH3, FAM135B, EGFR, cyclin-dependent kinase inhibitor 2A (CDKN2A) and TERT] were found in the 92 identical genes with mutations (Figure S4). The unique amplifications of FGF12, FGF10, serine/threonine kinase 24 (STK24), RPTOR-independent companion of MTOR complex 2 (RICTOR), mitogen-activated protein kinase kinase kinase 13 (MAP3K13), FGF14, ETS variant transcription factor 5 (ETV5), retinoid X receptor alpha (RXRA), and TNF superfamily member 13b (TNFSF13B) were discovered in the SCLC component.
of CSCLC, whereas amplifications of SOX2, TERT, NKX2-1, SDHA, FGF3, FGF4, FGF19, CCND1, CCNE1, AKT2, AKT1, X-ray repair cross complementing 3 (XRCC3), TNK2, serine/threonine kinase 11 (STK11), RAD54B, PRKCI, Rac family small GTPase 1 (RAC1), PMS1 homolog 2, and mismatch repair system component (PMS2) were found in both components of CSCLC (Figure S4).

However, when analyzing the mutated genes between 6 CSCLC cases containing AD and SCLC and 6 CSCLC cases containing SCC and SCLC, we found that 137 mutations were associated with 111 genes in 6 CSCLC cases expressing AD and SCLC, while 144 mutations were associated with 113 genes in 6 CSCLC cases expressing SCC and SCLC (Figure S1C and online material: https://

Figure 4 The biological process and the pathways of the mutated genes in different components predicted by the Database for Annotation, Visualization and Integrated Discovery (DAVID). (A,B) Images of the biological processes of the mutated genes in the combined small cell lung cancer (CSCLC) and pure small cell lung cancer (SCLC). (C,D) Images of signaling pathways involved in the mutated genes in combined small cell lung cancer (CSCLC) and pure SCLC.
Moreover, there were only 13 common mutations and 32 identical genes with mutations in both groups. The proportion of common mutations and identical genes with the mutation was 9.49% and 28.83% for CSCLC cases with AD and SCLC components respectively, and 9.03% and 28.32% for CSCLC cases with SCC and SCLC components, respectively (Figures S1C, S2C). Interestingly, amplifications of TERT, AKT1, XRCC3, SDHA, DOT1-like histone lysine methyltransferase (DOT1L), STK11, RAC1, PMS2, caspase recruitment

Figure 5 The biological process and the pathways of the mutated genes in different components predicted by the Database for Annotation, Visualization and Integrated Discovery (DAVID). (A,B) Images of the biological processes of the mutated genes in the non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) components of combined small cell lung cancer (CSCLC). (C,D) Images of the signaling pathways of the mutated genes in NSCLC and SCLC components of CSCLC.
domain family member 11 (CARD11), NF513B, and src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites (SRMS) were found only in CSCLC containing AD and SCLC components, while amplifications of SOX2, PIK3CA, RAD54B, PRKCI, FGFR4, FGFR3, FGFR19, CCND1, paired box 5 (PAX5), nuclear factor I B (NFIB), MET proto-oncogene, receptor tyrosine kinase (MET), MAP3K13, KLHL6, FA complementation group G (FANC G), ETV5, EMSY transcriptional repressor, BRCA2-interacting protein (EMSY), and CDKN2B were only detected in CSCLC containing SCC and SCLC components (Figure S5). In addition, when eliminating the mutated genes of the SCLC component, we discovered that 84 mutations were correlated with 65 genes in the AD components, and 89 mutations were associated with 73 genes in the SCC components (online material: https://cdn.america.ms/cn/static/public/tlcr-20-1099-1.docx and https://cdn.america.ms/cn/static/public/tlcr-20-1099-2.docx); of these only 4 common mutations and 13 identical genes with mutations were detected. Meanwhile, the rate of common mutations and identical genes with mutations was 4.76% and 20.00% for AD, respectively, and 4.49% and 17.81% for SCC, respectively (Figures S1D,S2D). Moreover, the amplifications of TERT, NOTCH3, DOTIL, AKT1, XRCC3, TNK2, STK11, SDHA, RXRA, RAC1, PMS2, PDCD1, NOTCH1, FGFR3, CCNE1, and CARD11 were only examined in the AD component. Meanwhile, the amplifications of SOX2, CDKN2A, FGFR4, FGFR3, FGFR19, CCND1, RAD54B, PRKCI, PRK3CA, PAX5, NFIB, MYC, MET, FANCG, and EMSY were found exclusively in the SCC component (Figure S6).

The mutational landscape in AD and SCLC components of CSCLC

Among the 12 CSCLC cases examined with NGS, six samples showed AD and SCLC features. Further analysis demonstrated that 84 mutations correlated with 65 genes in the AD component of CSCLC, and 111 mutations were linked with 93 genes in the SCLC component of CSCLC (Figure S1E and online material: https://cdn.america.ms/cn/static/public/tlcr-20-1099-1.docx and https://cdn.america.ms/cn/static/public/tlcr-20-1099-2.docx). GO and KEGG analysis indicated that certain common biological processes and classical signaling pathways were present in both components (Figure S7A,B,C,D). Further analysis indicated that 58 common mutations and 47 identical genes with mutations were detected in both components, respectively accounting for 52.25% and 50.54% of the SCLC components, and 69.05% and 72.31% of the AD components (Figures S1E,S2E). When analyzing the individual sample, the respective proportion of common mutations in case 1 to case 6 was 71.88%, 50.00%, 83.33%, 92.31%, 76.47% and 33.33% in AD components, and 56.10%, 26.67%, 71.43%, 75.00%, 56.52% and 20.00% in the SCLC components (Figure S1G). As displayed in Table S5, five RB1 common mutations were detected in five SCLC and AD components, followed by TP53 [4] common mutations.

The mutational landscape in SCC and SCLC components of CSCLC

The result of an examination of 6 samples with SCC and SCLC features showed that 89 mutations were associated with 73 genes in the SCC components and 110 mutations were related to 91 genes in the SCLC components (online material: https://cdn.america.ms/cn/static/public/tlcr-20-1099-1.docx and https://cdn.america.ms/cn/static/public/tlcr-20-1099-2.docx). Additionally, some common biological processes and classical signaling pathways were evident in both components as demonstrated by GO and KEGG analysis (Figure S8A,B,C,D). Moreover, 54 common mutations and 51 identical genes with mutations were detected in both components, respectively occupying 49.09% and 56.04% of the SCLC components, and 60.67% and 69.86% of the SCC components (Figure S1F). As shown in Figure S1H, the proportion of common mutations (case 1 to case 6) was 80.00%, 76.92%, 90.91%, 57.14%, 66.67%, and 4.35%, respectively in the SCC components and 95.24%, 38.46%, 45.45%, 20.00%, 53.85%, and 8.33% in the SCLC components, respectively. Additionally, five TP53 common mutations were discovered in five SCLC and SCC components, followed by RB1, SOX2, CDKN2A, FGFR19, etc. (Table S6).

TMB analysis

In terms of TMB, we identified that TMB in patients with CSCLC (8.89±4.93) was lower than that in patients with pure SCLC (10.70±9.49) (Figure S9A), and TMB in the SCLC component and NSCLC component of CSCLC was 8.93±4.965 and 8.86±5.20, respectively (Figure S9B). However, this difference was not significant. A previous
study showed that the DDR pathway associated with platinum sensitivity was related with TMB level (27). In current study, the level of TMB in pure SCLC patients with genetic mutation in DDR pathway was higher than that in patients without a genetic mutation in DDR pathway (P=0.008). Furthermore, the level of TMB in CSCLC patients with a genetic mutation in DDR pathway was higher, but not significantly so (P=0.711) (Figure S9C). Further analysis concerning the different components of CSCLC revealed that the level of TMB in the SCLC and NSCLC components with genetic mutation was elevated (Figure S9D); however, no significant difference was found. We then analyzed the role of TMB and DDR status on prognosis. Considering the limited number of CSCLC cases, the data of pure SCLC was used. We found there to be no significant differences in pure SCLC cases with different TMB and in pure SCLC cases receiving chemotherapy with different DDR status (Figure S9E,F).

Discussion

SCLC, an aggressive malignancy with frequent recurrence and poor prognosis cancer, accounts for about 15% of all lung cancers. In 1999, a deeper understanding of SCLC had led to the International Association for the Study of Lung Cancer (IASLC) to classify SCLC into two subtypes: pure SCLC and CSCLC (28). It has been generally understood that the NSCLC components of CSCLC might cover one or more different elements (usually SCC, AD, LCNC, and so on). Previous research has revealed that CSCLC constitutes about 13% of autopsy specimens of SCLC and 12–26% of surgical specimens of SCLC (29-31). In the current study, the incidence of CSCLC within resected specimens was 16%, and the prognosis for CSCLC was worse than that for pure SCLC. Further NGS results indicated that TP53 and RB1 were the most frequent mutated genes in both the CSCLC and pure SCLC groups. Moreover, fewer than 10% common mutations were found in both CSCLC and pure SCLC, while more than 50% common mutations were detected in both SCLC and NSCLC components of CSCLC, which may explain the tumor heterogeneity and common ancestral cells.

It is well known that the accumulation of sequential genomic alterations, as discovered by diverse methods, including loss of heterozygosity (LOH) analysis, comparative genomic hybridization (CGH), and DNA or whole-genome sequencing, leads to the tumorigenesis and progression of SCLC (9,22,32,33). A previous study demonstrated that allelic loss manifesting as LOH can be considered to result from the inactivation of a tumor suppressor via point mutations or other mechanisms and is associated with tumorigenesis (32). Murase et al. observed the role of point mutation in TP53 gene and allelic deletion of chromosome 3p in SCLC development (22). Based on the NGS technique, a recent study found TP53 to be the most frequently mutated gene, followed by RB1. Further analysis has suggested that the mutations of TP53, RB1, capping protein inhibiting regulator of actin dynamics (KIAA1211), and collagen type XXII alpha 1 chain (COL22A1) are implicated in G-protein-coupled receptor signaling and that high levels of delta-like non-canonical notch ligand 1 (Dlk1) and achaete-scute family BHLH transcription factor 1 (Ascl1) contribute to low notch pathway activity (10,34). These findings have identified many of the key mutated genes and several of the significant signaling pathways involved in SCLC that may provide candidate therapeutic targets for this lethal cancer.

Further analysis concerning the different components of CSCLC revealed that the level of TMB in the SCLC and NSCLC components with genetic mutation was elevated (Figure S9D); however, no significant difference was found. We then analyzed the role of TMB and DDR status on prognosis. Considering the limited number of CSCLC cases, the data of pure SCLC was used. We found there to be no significant differences in pure SCLC cases with different TMB and in pure SCLC cases receiving chemotherapy with different DDR status (Figure S9E,F).
growth and proliferation after incurring genetic mutations (22,40,41). Murase et al. found that the components of SCLC and SCC of CSCLC showed the same TP53 mutations and chromosomal abnormalities, which indicated that they might derive from the same clone before the TP53 mutations and chromosomal abnormalities occur (22). Wagner et al. suggested that the components of SCLC and NSCLC in CSCLC displayed a similar immunophenotype and neuroendocrine differentiation, and thus might share a clonal precursor closely related to SCLC (41). Additionally, other researchers have put forward the hypothesis that the various components of CSCLC might grow in neighbor regions independently and then come together within the primary tumor (23,42). When comparing the mutations of CSCLC and pure SCLC in the current study, there were more than 50% of identical genes with mutations present in both groups, including the top frequent mutations of TP53, RB1, TERT, FAM135B, NOTCH family genes, and EAT family genes. This phenomenon may evince the close association between the development of CSCLC and pure SCLC.

It is generally accepted that the different components of a tumor might share a common clonal origin owing to the high frequency of shared mutations. Hepatocellular-cholangiocarcinoma (H-ChC), a rare subtype of liver cancer, is composed of hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA). Wang et al. reported that a number of common mutations exist in the HCC and iCCA components of H-ChC and concluded that the various components of H-ChC derive from a common clonal origin (26). Similarly, Zhao et al. found that the SCC and SCLC components of CSCLC, which shared a substantial number of common mutations, might have the same clonal origin (28). However, only three cases and a limited DNA panel were used in the analysis. In our current study, 10 TP53 and 7 RB1 common mutations were identified in 12 cases of CSCLC, suggesting that the CSCLC components might derive from common precursors. In addition, more than 50% of the common mutations were detected in the NSCLC and SCLC components of CSCLC. Additionally, we discovered that the proportion of common mutations in the AD and SCLC components of CSCLC, including RB1, TP53, EGFR, and TERT, surpassed 50% in AD and SCLC. A similar phenomenon was found in CSCLC containing SCC and SCLC, with the frequently mutated genes being TP53, RB1, and SOX2. We thus speculate that the different components of CSCLC may be derived from the same pluripotent single clone due to key mutations of TP53 and RB1 as well as a high percentage of common mutations.

A previous study in a mouse model with various genetic alterations established by Ferone et al. revealed that SOX2 was pivotal for the shift to SCC from the original cells (38). In a comprehensive genomic study using NGS technologies to characterize 36 primary SCLC tumors and 17 SCLC cell lines, SOX2 amplification was found in approximately 27% of samples (43). Amplified SOX2 was first characterized as a driver oncogene in squamous lung cancer (44). SOX2 is a member of a large family of transcription factors involved in the maintenance of embryonic stem cells, induction of pluripotent stem cells, and lung cancer development, including the SCLC subtype (45). Meanwhile, TP53, EGFR, TERT and others were found to correlate with the development of AD (46,47). In contrast to the more common mutations mentioned above, less than 10% of the common mutations and 30% of the identical genes with mutations were found both in CSCLC cases with the AD and SCLC components and CSCLC cases with the SCC and SCLC components. Meanwhile, less than 5% of the common mutations and 20% of the identical genes with mutations were found in both the AD and SCC components. Notably, upon comparing the genes between AD and SCC, mutations of TERT, EGFR, NKX2-1, and SDHA were unique to AD, while mutations of SOX2, CDKN2A, and FAM135B were unique to SCC. We hypothesize that the different NSCLC components may undergo a separate progression from the primary clones, and the distinct genetic mutations and heterogeneity of the tumor may be responsible for this result.

Conclusions

Overall, our study identified no significant difference in the distribution of clinico-pathologic characteristics between the CSCLC and the pure SCLC group. However, the prognosis of CSCLC was worse than that of pure SCLC. Additionally, the results of NGS revealed that CSCLC and pure SCLC had a similar mutational profile. Further analysis revealed that different components of CSCLC might derive from the same pluripotent single clone. Nevertheless, little is known concerning the cellular and molecular abnormalities underlying CSCLC development, progression, and resistance to treatments.

Our study provides novel insights into the molecular features of CSCLC; however, further research is needed to confirm our findings. SCLC and CSCLC are heterogeneous...
diseases, characterized by genomic alterations targeting a broad variety of genes. It has been widely established that genomic analyses in tissue samples could be limited by tumor heterogeneity. In contrast to tissue biopsy, liquid biopsy could provide a more complete representation of the entire genomic landscape by including primary tumor and multiple metastatic sites. Moreover, molecular profiling on liquid biopsy can be performed at various points, thus potentially offering a dynamic portrait of genomic changes during the course of disease. Therefore, more studies focusing on the use of liquid biopsy are needed to identify novel genetic alterations involved in CSCLC development which can potentially serve as targets for novel therapeutic agents.

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Footnote

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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. This study was approved by the Institutional Review Board of Shanghai Pulmonary Hospital (ethical approval number: K18-066). Moreover, all procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was taken from all the patients.

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