Heliyon 6 (2020) e04439

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

# Detection of somatic *TP53* mutation in surgically resected small-cell lung cancer by targeted exome sequencing: association with longer relapse-free survival

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ARTICLE INFO

*Keywords:*
Bioinformatics
Genetics
Cancer research
Respiratory system
Clinical genetics
Surgery
Oncology
Clinical research
Small-cell lung cancer
Mutation
Next-generation sequencing
TP53

ABSTRACT

*Objectives:* Few reports have explored clinical biomarkers, including those identified by targeted exome sequencing (TES) of surgically resected small-cell lung cancer (SCLC) and correlation with patient survival.
*Patients and methods:* We collected formalin-fixed paraffin-embedded tumor samples from 127 patients with SCLC who had undergone surgery and analysed nonsynonymous somatic gene mutation profiles by TES of 26 cancer-related genes using next-generation sequencing (NGS) and web databases (UMIN Registration No. 000010117).
*Results:* We detected 38 nonsynonymous somatic *tumor protein p53* (*TP53*) mutations in 43 (54.4%) patients. Among these *TP53* lesions, we identified clinically relevant mutations including those encoding Y220C, R248W, R249M, M237I, and R273L substitutions in the p53 protein. These mutations have been reported to be associated with certain clinical outcomes or biology in other types of malignancies but not in SCLC. Moreover, non-synonymous somatic mutations of *TP53* were positively associated with relapse-free survival (RFS) (median, 17.33 months [95% confidence interval (CI), 3.86–30.79] in a mutation-positive group vs 10.39 months (6.96–13.82) in a mutation-negative group, p = 0.042). Multivariate analysis revealed that nonsynonymous

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https://doi.org/10.1016/j.heliyon.2020.e04439
Received 14 December 2019; Received in revised form 14 April 2020; Accepted 9 July 2020

somatic *TP53* mutation was an independent factor of prolongation of RFS (hazard ratio: 0.51, 95% CI: 0.29–0.89, p = 0.019) but not overall survival (OS).
*Conclusion:* These data suggested that TES may play a critical role for promoting reverse-translational studies, including investigations of the biology of *TP53* mutations in different stages of SCLC. Accumulation of the data using cancer panels with a broader range of genes, including *TP53*, is expected to be useful for future clinical applications for patients with SCLC.

## 1. Introduction

Small-cell lung cancer (SCLC) accounts for approximately 13–15% of all lung cancers [1, 2]. Overcoming SCLC remains a large obstacle due to the limited numbers of available treatments and the high proliferative index of this cancer. Thus, thorough exploration of novel treatment strategies is needed.

Recent discovery of relevant gene alterations in non-small-cell lung cancer (NSCLC) has accelerated the development of treatments for patients with NSCLC [3]. Indeed, a significant increase in survival was demonstrated in patients harboring tumors with such gene alterations and who received genotype-directed therapy [4]. In the field of SCLC, whole-genome or whole-exome sequence (WES) analysis using next-generation sequencing (NGS) systems has revealed that SCLC also harbors potential targets with gene alterations, including *SOX-2* amplification [5], mutations in genes responsible for histone modification [6, 7, 8], and changes genes encoding components of the PI3K/AKT/mTOR pathway [9], suggesting that novel treatment strategies directed to these targets have potential for treating patients with SCLC. Indeed, several ongoing clinical trials for the treatment of SCLC are examining the role of mutations in the genes encoding components of the PI3K/AKT/mTOR pathway in tumors [9]. Separately, experiments have shown that classification of gene copy-number aberrations in circulating tumor cells from pretreatment SCLC blood samples can predict chemosensitivity [10]. However, there is an ongoing debate regarding the utility of comprehensive whole-genome sequencing or WES in clinical use compared with targeted exome sequencing (TES) from the perspectives of data interpretation, time, and cost due to the high volume of information generated by NGS systems [11].

Recently, various TES studies using clinical samples from patients with SCLC have identified mutations for drug targets [12], prediction of response to immune checkpoint inhibitors [13], and gene mutation profiling for diagnosis [14]. A previous paper demonstrated that *TP53* mutation is associated with unfavorable overall survival (OS) in patients with limited disease (LD)-SCLC [15]. However, few reports have attempted to validate the clinical utility of TES using a number of surgically resected SCLC tumor specimens in combination with corresponding clinical data, including survival times.

Given these findings, the objective of the present study was two-fold. The first goal was to use our TES system to explore clinically meaningful somatic mutations, including drug targets. The second goal was to assess the relationship between mutation profiles and clinical variables including relapse-free survival (RFS) and/or OS. Together, these results were expected to address whether TES is applicable for clinical use and as an aid in establishing treatment strategies in individual patients with early-stage SCLC.

## 2. Patients and methods

### 2.1. Patient data

Our eligibility criteria allowed the inclusion of patients with primary SCLC who had undergone complete surgical resection of the primary lung tumor. The study represented patients subjected to surgery from January 2003 through January 2013 at the participating institutions, including either the Fukushima Investigative Group for Healing Thoracic Malignancy (FIGHT) or the Hokkaido Lung Cancer Clinical Study Group Trial (HOT). Written informed consent was obtained only from patients who were still alive at the time of data accrual (from February 2013 through January 2014).

The requirement for consent was waived if the patient had died or could not be contacted. In such cases, investigators of each participating institution were required to provide subjects with a written statement regarding the research in the outpatient department or via a website.

This study was registered with the University Hospital Medical Information Network (UMIN) Clinical Trials Registry as Identification Number UMIN000010117; this trial included immunohistochemistry, results of which were reported previously [16]. The study protocol was approved by the Institutional Review Boards of the respective participating institutions. All individual data were obtained from medical records and de-identified. Each tissue sample was anonymized by assigning a randomized code number. Stages were determined or reclassified according to the seventh edition of the tumor, node, metastasis (TNM) staging system [17].

This is a retrospective non-interventional genetic association study. Thus, we used the STREGA checklist when writing our report [18].

### 2.2. Samples

All the cases that were included in the present study met the following criteria: a complete surgical resection of the primary tumors had been performed; and a central re-review confirmed a pathological diagnosis of SCLC or combined SCLC according to the 2004 World Health Organization classification [19]. Each formalin-fixed paraffin-embedded (FFPE) tissue block was cut so as to yield five sections with 20-μm thicknesses, obtained as a paraffin roll, for use in NGS. Total DNA was obtained from each of the samples. Preparation of DNA and NGS analysis were performed at the Department of Translational Pathology, Hokkaido University Graduate School of Medicine. This is a retrospective observational study. In addition, the number of SCLC patients who underwent surgery is generally limited. Thus, we did not set an appropriate sample size for this study, and instead we attempted to collect as many samples as possible that annotated to clinical data from the institutions.

### 2.3. TES and mutation profiling

Genomic DNA was extracted from FFPE tissues using QIAamp DNA FFPE Tissue Kits (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. The quality of genomic DNA was assessed using Qubit dsDNA BR assay kits, a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and GeneRead DNA QuantiMIZE Assay Kits (Qiagen). The TruSight Tumor Sequencing Panel (Illumina) was used for library preparation with genomic DNA following the manufacturer's instructions. The quality of the libraries was assessed using an Agilent 2100 bioanalyser (Agilent Technologies, Santa Clara, CA, USA) with Agilent DNA 1000 Kits (Agilent Technologies). The libraries were sequenced using MiSeq (Illumina, San Diego, CA, USA) to produce 150-bp paired-end reads. The target exons of 26 cancer-related genes (Table 1) were loaded on the TruSight Tumor Sequencing Panel (Illumina), which allows detection of hotspot somatic mutations across 14 Kb of exons (21 Kb total length of exons and introns) in genes that are commonly mutated across multiple forms of cancer. The 26 genes selected under the supervision of the College of American Pathologists and The National Comprehensive Cancer Network were all cancer

**Table 1.** List of genes on TruSight Tumor Sequencing Panel.

| | | | | |
|---|---|---|---|---|
| *AKT1* | *EGFR* | *GNAS* | *NRAS* | *STK11* |
| *ALK* | *ERBB2* | *KIT* | *PDGFRA* | *TP53* |
| *APC* | *FBXW7* | *KRAS* | *PIK3CA* | |
| *BRAF* | *FGFR2* | *MAP2K1* | *PTEN* | |
| *CDH1* | *FOXL2* | *MET* | *SMAD4* | |
| *CTNNB1* | *GNAQ* | *MSH6* | *SRC* | |

Gene products: *ALK*, anaplastic lymphoma kinase; *APC*, adenomatous polyposis coli; *CDH1*, cadherin 1; *CTNNB1*, catenin beta 1; *EGFR*, epidermal growth factor receptor; *FBXW7*, F-box and WD repeat domain containing 7; *FGFR2*, fibroblast growth factor receptor 2; *FOXL2*, forkhead box L2; *GNAQ*, guanine nucleotide binding protein, Q polypeptide; *GNAS*, guanine nucleotide binding protein, alpha stimulating; *MSH6*, MutS homolog 6; *PDGFRA*, platelet-derived growth factor receptor, alpha; *PIK3CA*, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha; *PTEN*, phosphatase and tensin homologue; *STK11*, serine/threonine kinase 11, also known as liver kinase B1 (LKB1); *TP53*, tumor protein P53.

related. Base calling of variant frequency (VF) was performed using Miseq Reporter v2.3 (Illumina) with the default parameter of VF >3.0%. The paired-end sequence reads that passed the quality-control metrics determined by the pipeline were included in the analysis.

BAM files obtained from Miseq Reporter v2.3 were processed by the BioReT System (Amelieff, Tokyo, Japan) for analysis of mutations. In the BioReT System, BAM files were realigned and recalibrated with the Genome Analysis Toolkit (GATK) (version 1.6.13), using RealignerTargetCreator, IndelRealigner, CountCovariates, and TableRecalibration. Single-nucleotide variants (SNVs) and small indels were detected using the GATK UnifiedGenotyper, followed by filtering for low-quality variants using the GATK VariantFiltration. All analysis was performed with the default settings except for the minIndelFrac parameter for indel call using GATK UnifiedGenotyper, which was set to 0.05. After variant detection, VCF files were annotated by the SnpEff genetic variant annotation and effect prediction toolbox (version 4.0). Information from

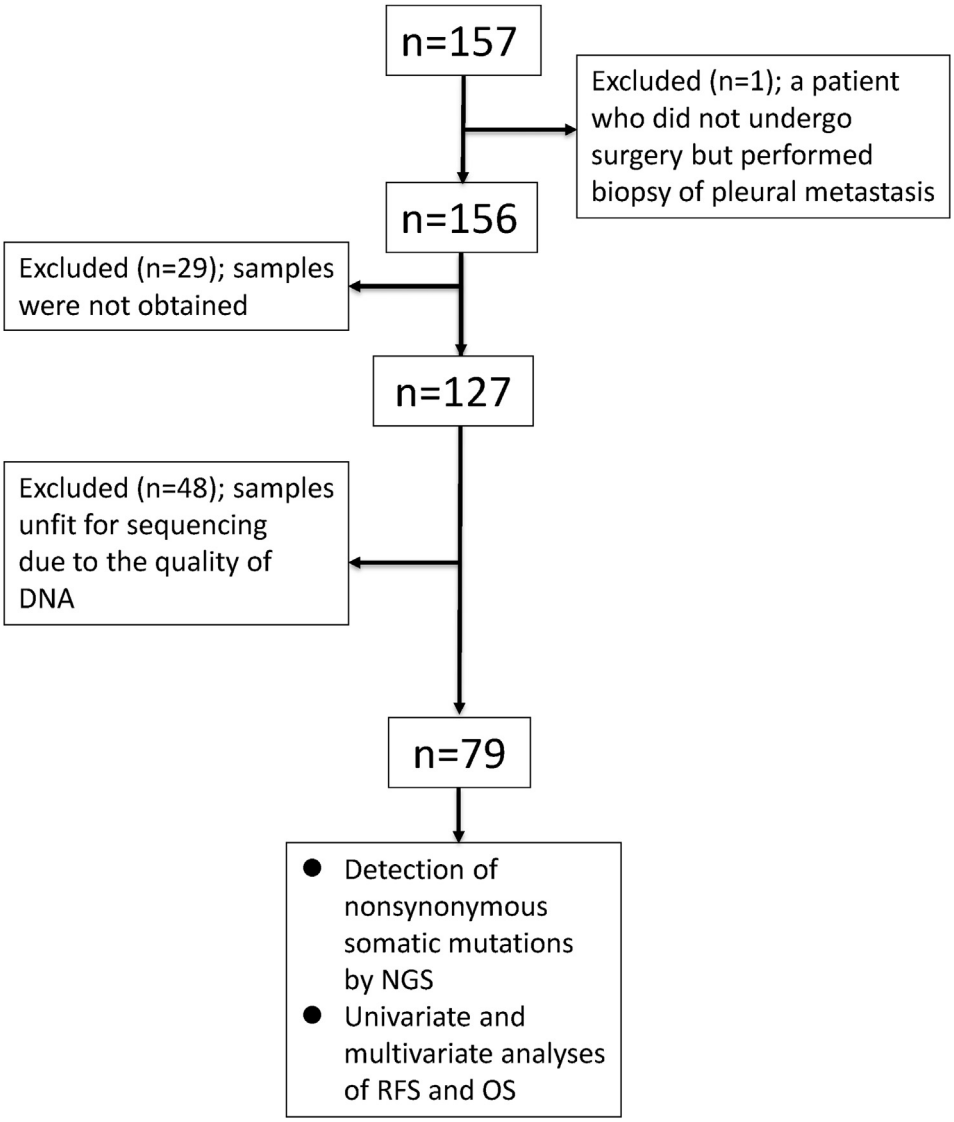


**Figure 1.** Flow chart diagram. NGS, next-generation sequencing; RFS, relapse-free survival; OS, overall survival.

the Catalogue of Somatic Mutations in Cancer (COSMIC) database (version 72) and IntOGen (Integrative Onco Genomics, version 1412) were used to annotate the VCF sequences using SnpSift, a package tool of SnpEff, and variants on targeted genes were extracted. SNVs were limited to protein-altering mutations at ≥10% VF with read-depths of >100. The resulting mutations detected by our TES were stratified into three categories: i) major mutations that were annotated in the COSMIC database and were recognized as driver genes by IntOGen, ii) sub-major mutations that were annotated only in COSMIC, and iii) minor mutations that were not annotated in either COSMIC or IntOGen. All sub-major mutations were synonymous mutations, and all minor mutations were not annotated in the COSMIC database. Therefore, the lesions that were categorized as sub-major and minor mutations were excluded from further consideration in the present study. Next, potential germline variants were manually excluded by reference to gnomAD (http://gnomad.broadinstitute.org), a web database that spans 125748 exomes and 15708 genomes from individuals. In an attempt to remove additional germline mutations and to determine the pathogenicity, evidence level, clinical relevance, and description of putative SCLC-associated somatic gene mutations, we consulted several web databases, including ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), CIViC (Clinical Interpretation of Variants in Cancer, https://civic.genome.wustl.edu/#/home), OncoKB (A Precision Oncology Knowledge Base, https://oncokb.org/), IARC TP53 database (p53.iarc.fr/TP53GeneVariations.aspx), and ICGC Data Portal (https://dcc.icgc.org/). The day of last data acquisition from these web databases was March 31, 2020. The analyst (H.M. in the Acknowledgements) was not informed of any data with regard to patient survival and other clinical results.

### 2.4. Statistical analysis

Univariate and multivariate Cox proportional hazard model analyses were performed to examine the association between clinical variables, including gene mutations, and either the RFS or OS. For factors that were significant in univariate analysis, we confirmed the Spearman's rank correlation coefficient ($r_s$) and avoided entering multiple variables with a high correlation ($r_s \geq 0.6$) and similar significance. RFS was calculated from the date of surgery to the date of first disease recurrence or death. OS was calculated from the date of surgery to the date of death. Patients who had survived through the observation period were censored at the date for which last available information on status was available. Survival curves were estimated using the Kaplan–Meier method, and differences in survival distributions were evaluated using the log-rank test. All statistical analyses were performed using SPSS version 20 (IBM Corporation, Armonk, NY, USA). A $p$ value of $<0.05$ was considered statistically significant.

### 2.5. Data statement

Raw data of major mutations detected by BioReT System were uploaded in Mendeley Data, V1 (https://doi.org/10.17632/pczf7nwxp8.1). Synonymous and potential germline mutations were omitted because those mutations were not allowed to be disclosed according to the initial research plan which had been submitted to Institutional Review Boards of the respective participating institutions and the UMIN Clinical Trials Registry.

## 3. Results

### 3.1. Patient characteristics

A flow chart schematic diagram of the study is provided in Figure 1. Between January 2003 and January 2013, 157 patients were enrolled

**Table 2.** Demographic and clinical characteristics of patients included in this study.

| Variables | Patients (n = 79) | |
|---|---|---|
| | No. | % |
| Age, median (range in years) | 69 (44–85) | |
| Sex | | |
| Female | 22 | 27.8 |
| Male | 57 | 72.2 |
| Smoking status | | |
| Never-smoker | 6 | 7.6 |
| Smoker (current or former) | 68 | 86.1 |
| Unknown | 5 | 6.3 |
| ECOG PS | | |
| 0 | 52 | 65.8 |
| 1 | 23 | 29.1 |
| Unknown | 4 | 5.1 |
| Maximum tumor diameter, median (mm) | 21 (9–64) | |
| Histology | | |
| SCLC | 62 | 78.5 |
| Combined SCLC | 17 | 21.5 |
| Clinical stage (TNM, version 7·0) | | |
| IA | 51 | 64.6 |
| IB | 8 | 10.1 |
| IIA | 11 | 13.9 |
| IIB | 3 | 3.8 |
| IIIA | 5 | 6.3 |
| IIIB | 1 | 1.3 |
| Pathologic stage (TNM, version 7·0) | | |
| IA | 31 | 39.2 |
| IB | 20 | 25.3 |
| IIA | 10 | 12.7 |
| IIB | 2 | 2.5 |
| IIIA | 13 | 16.5 |
| IIIB | 1 | 1.3 |
| IV | 2 | 2.5 |
| Adjuvant chemotherapy | | |
| Yes | 49 | 62.0 |
| No | 29 | 36.7 |
| Unknown | 1 | 1.3 |
| Comorbidity or past history | | |
| Interstitial pneumonitis | 9 | 11.4 |
| Other types of cancer | 25 | 31.6 |
| Serum level of LDH | | |
| < ULN | 58 | 73.4 |
| ≥ ULN | 21 | 26.6 |
| Approach | | |
| VATS | 45 | 57.0 |
| Open surgery | 34 | 43.0 |
| Type of surgical resection | | |
| Lobectomy | 55 | 69.6 |
| Partial resection | 22 | 27.8 |
| Pneumonectomy | 2 | 2.5 |
| PCI | | |
| Yes | 6 | 7.6 |
| No | 71 | 89.9 |
| Unknown | 2 | 2.5 |

ECOG PS, Eastern Cooperative Oncology Group performance status; SCLC, small-cell lung cancer; TNM, tumor-node-metastasis; LDH, lactate dehydrogenase; ULN, upper limit of normal range; TNM, tumor-node-metastasis; VATS, video-assisted thoracoscopic surgery; PCI, prophylactic cranial irradiation.

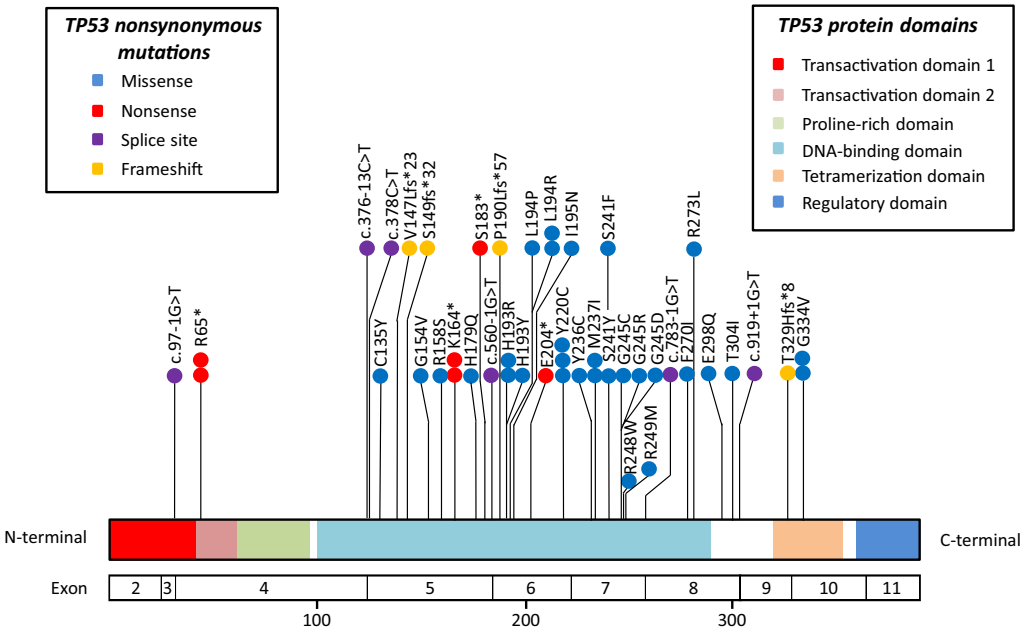


**Figure 2.** Distribution of 38 nonsynonymous somatic *TP53* mutations identified in this study. Missense mutations are indicated in blue, nonsense mutations in red, splice site mutations in purple, and frameshift (fs) mutations in orange. Each circle represents a detected mutation. Numbers in the white bar denote the respective exon, and numbers below the bar show corresponding amino acid sequence. For mutation designations, single-letter abbreviations are used for amino acids, except where C > T and G > T notations are used to indicate nucleotide substitutions; an asterisk indicates a stop codon.

from 17 institutions. One hundred twenty-seven tumor samples were obtained from 16 institutions. Of the samples, 48 (37.8%) were unfit for sequencing due to the poor quality of the DNA. Baseline characteristics of the remaining 79 patients are listed in Table 2. Median age was 69 years, 22 (27.8%) patients were female, and 6 (7.6%) were never-smokers. Eastern Cooperative Oncology Group performance status was 0 in 52 (65.8%) patients. The median maximum tumor diameter was 21 mm. The numbers of patients with SCLC and combined SCLC were 62 (78.5%) and 17 (21.5%), respectively. In terms of pathological stage, 31 cases were IA, 20 were IB, 10 were IIA, 2 were IIB, 13 were IIIA, 1 was IIIB, and 2 were IV. Adjuvant chemotherapy was conducted in 49 (62.0%) patients, including 6 patients who received adjuvant chemoradiotherapy.

### 3.2. Mutations detected by TES

DNA libraries of 79 samples (62.2%) were successfully subjected to NGS. We detected 38 nonsynonymous somatic *TP53* mutations in 43 (54.4%) patients. A summary of the detected and confirmed mutations in *TP53* is provided in Figure 2, and the same lists are shown with clinical data in Table 3 and Table 4. The vast majority of *TP53* mutations corresponded to missense mutations within the DNA-binding domain of the protein, irrespective of the degree of VF. As shown in Table 3, the *TP53* mutations that we identified included clinically relevant mutations that encoded proteins with Y220C, R248W, R249M, M237I, and R273L substitutions. All of these mutations have been implicated (by clinical or preclinical evidence; as described in CIViC) in other types of malignancies but not in SCLC.

The detailed results for five pathogenic or potentially pathogenic somatic mutations in genes other than *TP53* that were detected in 5 patients are shown in Table 5 with corresponding clinical data. The mutations included an *AKT1* E17K mutation that has been reported to render tumors sensitive to AZD5363 (capivasertib), an Akt inhibitor [20]; an *EGFR* E746_A750del mutation that has been reported to render tumors sensitive to an EGFR tyrosine kinase inhibitor [21], a *FBXW7* R505G mutation that is likely pathogenic [22, 23]; and *KRAS* G12D and Q61H mutations, both of which are associated with shorter progression-free survival and overall survival by anti-EGFR antibody in colorectal cancer [24, 25] and responsiveness to MEK inhibitor in combination with cyclin dependent kinase 4/6 inhibitor [26, 27]. The four of the five patients who harbored these gene mutations were those with combined small-cell carcinoma.

### 3.3. Association between somatic TP53 mutations and RFS or OS in SCLC

The median follow-up time of 79 patients was 24.13 months (range, 0.36–119.97). Nonsynonymous somatic mutations of *TP53* were positively associated with RFS [median, (95% confidence interval): 17.33 months (3.86–30.79) in mutation-positive group vs 10.39 months (6.96–13.82) in mutation-negative group, $p = 0.042$]. The OS was nominally but not statistically longer in the mutation-positive group compared with mutation-negative group [median, (95% confidence interval): 44.88 months (20.00–69.76) in mutation-positive group vs 29.06 months (21.64–36.49) in mutation-negative group, $p = 0.127$] (Figure 3). Univariate analysis of RFS revealed that this parameter was statistically larger in patients who underwent lobectomy, those with p-stage IA, those who underwent adjuvant chemotherapy, and those who harbored a *TP53* mutation (Table 6). We did not identify any confounding factors among these four variables using Spearman's rank correlation coefficient (data not shown). Multivariate analysis using the four variables demonstrated that *TP53* mutation was an independent factor of prolongation of RFS (hazard ratio: 0.51, 95% confidence interval: 0.29–0.89, $p = 0.019$) (Table 7). However, univariate and multivariate analysis revealed that OS was not significantly associated with *TP53* mutation (Table 8 and Table 9).

To examine the association between *TP53* mutation and response to chemotherapy, we generated Kaplan–Meier curves for patients with and without *TP53* mutations, performing this analysis separately for patients who did (Figure 4A) and did not (Figure 4B) receive adjuvant chemotherapy. RFS was nominally, but not significantly, prolonged in patients with *TP53* mutations compared with those lacking *TP53* mutations, both in the cohort of patients who underwent adjuvant chemotherapy (median: 54.74 months [95% CI, NR (not reached)–NR] vs 12.33 months [95% CI, 3.33–21.33], p = 0.070) and in the cohort of patients who did not receive adjuvant chemotherapy (median: 15.42 months [95% CI, 11.63–19.21] vs 6.90 months [95% CI, 1.63–12.17], $p = 0.415$).

**Table 3.** Clinically relevant or potentially clinically relevant somatic mutations of *TP53* mutation and clinical features in our cohort.

| Exon | Mutation | Variant pattern | VF | COSMIC ID | ClinVar annotation | CIViC evidence level | OncoKB description | Clinical relevance | Histology | Sex | Age (years) | Smoking status | p-stage |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 | Y220C | missense | 0.28713 | 10758 | Likely pathogenic | C | Oncogenic | TP53-independent response to bortezomib in breast cancer | SCLC | M | 78 | unknown | IA |
| | | | 0.72078 | | | | | | SCLC | M | 76 | ever | IB |
| | | | 0.84699 | | | | | | w/Sq | M | 60 | ever | IV |
| 7 | M237I | missense | 0.44355 | 10834 | Likely pathogenic | D | Likely oncogenic | Resistance to chemotherapeutic agents in AML cell lines | SCLC | M | 65 | ever | IA |
| | | | 0.69928 | | | | | | SCLC | M | 62 | ever | IA |
| 7 | R248W | missense | 0.2816 | 10656 | Likely pathogenic | B | Likely oncogenic | Worse prognosis in breast cancer | SCLC | M | 85 | ever | IB |
| 7 | R249M | missense | 0.2845 | 43871 | Likely pathogenic | B | Likely oncogenic | Better response to doxorubicin in breast cancer | SCLC | M | 71 | never | IA |
| 8 | R273L | missense | 0.22636 | 3675521 | Likely pathogenic | C | Likely oncogenic | Refractory to platinum-based chemotherapy and shorter time to disease progression and reduction of survival in ovarian cancer | w/La | M | 61 | unknown | IA |

VF, variant frequency; Age, age at diagnosis; p-stage, pathological stage; SCLC, small-cell lung cancer; w/Sq, combined with squamous cell carcinoma; AML, acute myeloid leukemia; w/La, combined with large cell carcinoma; CIViC evidence level B, clinical: clinical trials or other primary patient data supports association; CIViC evidence level C, case study: individual case reports from clinical journals; CIViC evidence level D, preclinical: *in vivo* or *in vitro* models support association.

## 4. Discussion

We analyzed hotspot mutations in 26 cancer-related genes using a TES system in tumors from patients with surgically resected SCLC. Specifically, we found 54.4% of the patients harbored tumors containing *TP53* nonsynonymous somatic mutations. Among these lesions, we found several clinically relevant somatic mutations. However, the development of treatments that target TP53 has not been clinically successful. In addition, as shown in Table 5, only five pathogenic or potentially pathogenic somatic mutations in genes other than *TP53* were identified. These results suggested that the limited number of genes included in the cancer panel in the present study were not sufficient for practical identification of novel drug targets.

All of the five clinically relevant mutations of *TP53*, which are shown in Table 3, have been reported to have a relationship with prognosis, sensitivity or insensitivity to chemotherapy. These previous *TP53* results were described in the literature in the context of acute myeloid leukemia (AML), or breast or ovarian cancer, but not in the context of SCLC.

The existence or the number of *TP53* mutations has been demonstrated to be associated with unfavorable OS in patients with LD-SCLC [15], lung adenocarcinoma [28], NSCLC [29, 30], and other malignancies [31, 32, 33]. However, in our study, nonsynonymous somatic mutations of *TP53* were positively associated with RFS and nominally with improved OS. The distinct associations between *TP53* mutations and survival may be attributable to basic TP53 biology.

*TP53* is known as a master transcription factor and critical tumor suppressor. Wild-type (WT) *TP53* in cancer has generally been described as an inducer of cell cycle arrest and apoptosis by cellular stress such as chemotherapy. In many cancer types, a strong correlation exists between the presence of *TP53* mutations and reduced responses to chemotherapeutic agents and, thus, a poor prognosis [34, 35]. We, therefore, examined whether chemotherapy might be associated with the shorter survival of patients with *TP53* mutation than those without in our cohort. However, there was no difference in RFS between patients with *TP53* WT and those with *TP53* mutation irrespective of chemotherapy (Figure 4), suggesting that the difference in RFS between patients with *TP53* WT and those with *TP53* mutation might be due to other factors.

An enormous amount of research has established multiple aspects of *TP53* functionality and its network in the context of cells [36]. We summarized the potential pathobiological factors of *TP53* which were expected and lacking in the present study.

i) *TP53* target: conversely, *TP53* has been demonstrated to regulate proteins that exert an anti-apoptotic potential. Anti-apoptotic *TP53* targets include genes related to DNA repair, cell cycle control, oxidative stress response, co-transcriptional factors, TP53-binding proteins, and MAPK signaling. The expression levels and duration of occupancy of these targets in tumor cells are context dependent [37].

ii) Tumor microenvironment: senescence-associated secretory phenotype (SASP) is a phenotype associated with senescent cells regulated by specific transcription factors including *TP53*, and can be responsible for chronic inflammation and age-linked diseases including cancer [38]. *TP53*-driven SASP in tumor stroma can create a tumor-suppressive immune milieu that influences the incidence of cancer [39, 40]; however, the SASP can mediate chronic inflammation and stimulate the growth and survival of tumor cells in a cell context-dependent manner [41, 42]. The *TP53* WT in our patients might induce the SASP, thereby creating an environment that promotes tumor proliferation.

iii) Cancer immunity: *TP53* regulates the expression of the natural-killer group 2, member D ligands, either positively or negatively as a transcriptional target through the upregulation of miR-34a. The miR34 family suppresses programmed death ligand 1 expression, an inhibitor of T cell activity [43]. These results suggest that exploring the difference in the tumor immune microenvironment within our SCLC samples with or without *TP53* mutation might be important.

**Table 4.** Nonsynonymous somatic mutations of TP53 detected using target exome sequence, potential clinical relevance, and clinical features.

| Exon | Mutation | Variant pattern | VF | COSMIC ID | ClinVar annotation | CiVIC evidence level | OncoKB description | Our cohort | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Histology | Sex | Age | Smoking status | p-stage |
| 3–4 | c.97-1G > T | Splice site | 0.12389 | 1610881 | NA | NA | NA | combined with la | F | 64 | ever | IA |
| 4 | R65* | Nonsense | 0.31438 | 1646878 | Pathogenic | NA | Likely oncogenic | SCLC | M | 74 | ever | IIIA |
| | | | 0.23908 | | | | | combined with ln | M | 76 | ever | IIA |
| 4–5 | c.376-13C > T | Splice site | 0.10526 | 44442 | NA | NA | NA | SCLC | M | 74 | ever | IA |
| 5 | c.378C > T | Splice site | 0.11648 | 44196 | NA | NA | NA | combined with sq | F | 68 | ever | IIIA |
| 5 | C135Y | Missense | 0.33231 | 10801 | Likely pathogenic | NA | Likely oncogenic | combined with ln | M | 76 | ever | IB |
| 5 | V147Lfs*23 | Frameshift | 0.34438 | 44698 | NA | NA | Likely oncogenic | SCLC | M | 70 | ever | IB |
| 5 | S149fs*32 | Frameshift | 0.48219 | 1324767 | NA | NA | Likely oncogenic | combined with ad | F | 63 | never | IIB |
| 5 | G154V | Missense | 0.38025 | 342245 | NA | NA | Likely oncogenic | SCLC | M | 52 | ever | IIIA |
| 5 | R158S | Missense | 0.80265 | 3970361 | NA | NA | Likely oncogenic | SCLC | M | 74 | ever | IA |
| 5 | K164* | Nonsense | 0.57135 | 10750 | NA | NA | Likely oncogenic | SCLC | F | 64 | ever | IB |
| | | | 0.63432 | | | | | SCLC | M | 60 | ever | IIA |
| 5 | S183* | Nonsense | 0.90281 | 10706 | Pathogenic | NA | Likely oncogenic | SCLC | F | 61 | ever | IB |
| 5 | H179Q | Missense | 0.75284 | 1649385 | NA | NA | Likely oncogenic | SCLC | F | 73 | never | IIIA |
| 6 | c.560-1G > T | Splice site | 0.45194 | 43841 | NA | NA | NA | SCLC | M | 58 | ever | IIIA |
| 6 | P190Lfs*57 | Frameshift | 0.29657 | 45320 | NA | NA | Likely oncogenic | SCLC | M | 73 | ever | IA |
| 6 | H193R | Missense | 0.52225 | 10742 | Likely pathogenic | NA | Likely oncogenic | SCLC | M | 74 | ever | IA |
| | | | 0.31318 | | | | | SCLC | M | 66 | ever | IB |
| 6 | H193Y | Missense | 0.33631 | 10672 | Likely pathogenic | NA | Likely oncogenic | SCLC | M | 66 | ever | IA |
| 6 | L194P | Missense | 0.28781 | 437527 | Likely pathogenic | NA | Likely oncogenic | SCLC | F | 58 | ever | IB |
| 6 | L194R | Missense | 0.46984 | 117647 | Likely pathogenic | NA | Likely oncogenic | SCLC | M | 71 | ever | IV |
| | | | 0.41548 | | | | | SCLC | M | 69 | ever | IA |
| 6 | I195N | Missense | 0.23674 | 44877 | Likely pathogenic | NA | Likely oncogenic | SCLC | F | 73 | unknown | IA |
| 6 | E204* | Nonsense | 0.38789 | 165087 | NA | NA | Likely oncogenic | combined with sq | M | 74 | ever | IB |
| 7 | Y236C | Missense | 0.13005 | 10731 | Likely pathogenic | NA | Likely oncogenic | combined with sq | F | 68 | ever | IIIA |
| 7 | S241F | Missense | 0.31855 | 10812 | Likely pathogenic | NA | Likely oncogenic | combined with ad | F | 63 | never | IIB |
| 7 | S241Y | Missense | 0.6879 | 10935 | Likely pathogenic | NA | Likely oncogenic | SCLC | M | 76 | ever | IA |
| 7 | G245C | Missense | 0.40776 | 11081 | Likely pathogenic | NA | Likely oncogenic | combined with ad | M | 64 | ever | IA |
| 7 | G245D | Missense | 0.77484 | 3388189 | Likely pathogenic | NA | Likely oncogenic | SCLC | M | 75 | ever | IA |
| 7 | G245R | Missense | 0.70946 | 10957 | Likely pathogenic | NA | Likely oncogenic | SCLC | M | 76 | ever | IIA |
| 8 | c.783-1G > T | Splice site | 0.63082 | 6913 | Likely pathogenic | NA | NA | SCLC | M | 64 | ever | IIIA |
| 8 | F270I | Missense | 0.69492 | 437484 | Likely pathogenic | NA | Likely oncogenic | SCLC | M | 69 | ever | IB |
| 8 | E298Q | Missense | 0.45481 | 45938 | NA | NA | NA | combined with sq | F | 53 | ever | IA |
| 8 | T304I | Missense | 0.58434 | 45128 | NA | NA | NA | SCLC | M | 77 | ever | IB |
| 9 | c.919+1G > T | Splice site | 0.84719 | 2744491 | Likely pathogenic | NA | NA | SCLC | M | 75 | ever | IIA |
| 9 | T329Hfs*8 | Frameshift | 0.7753 | 5002556 | NA | NA | Likely oncogenic | combined with sq | M | 75 | ever | IIA |
| 10 | G334V | Missense | 0.16953 | 11514 | NA | NA | Oncogenic | SCLC | M | 72 | ever | IB |
| | | | 0.53128 | | | | | combined with la | M | 62 | ever | IB |

VF, variant frequency; COSMIC, Catalogue of Somatic Mutations in Cancer; ID, identification; p-stage, pathological stage; NA, not available; la, large cell carcinoma; SCLC, small-cell lung cancer; ln, large cell neuroendocrine carcinoma; sq, squamous cell carcinoma; ad, adenocarcinoma. For mutation designations, single-letter abbreviations are used for amino acids, except where C > T and G > T notations are used to indicate nucleotide substitutions; an asterisk indicates a stop codon.

iv) Autophagy regulation: *TP53*-driven cellular senescence may be supported by activation of autophagy [44]. In some settings, autophagy has the potential to delay apoptosis by reducing the levels of the pro-apoptotic BH3-only protein PUMA [45]. The dual roles of autophagy in cancer, including tumor progression and promotion, are also cell reliant [46]. Autophagy regulation and related factors might affect the survival of our SCLC patients.

v) Cancer stem cell (CSC)-like features: CSCs are associated with aggressive cancer behavior, metastatic progression, resistance to therapy and relapse. $CD133^{+}$ cancer stem-like cells in SCLC are highly tumorigenic and resistant to chemotherapy [47, 48, 49]. Although *TP53* was previously reported to transcriptionally suppress CD133 expression [50], *TP53* may function with different transcription factors in colorectal cancer to maintain the stem cell properties [51], which may be independent of the tumor suppressor role of *TP53*. *TP53* WT in early-stage SCLC might correlate with the nature of CSC. Examining the expression of CSC-like markers including CD133 in combination with *TP53* WT/mutation, may be one of the methods for clarifying the reason for differences in RFS.

vi) Intratumor genetic heterogeneity: a previous report described that cancer cells with *TP53* WT and *TP53* mutation resided as different clusters in the same tumor sample of prostate cancer [52]. The use of surgical specimens, as in the present study, can avoid the clonal heterogeneity that is observed in small biopsy samples, an aspect that is a strength of our study.

This study has several limitations that require cautious interpretation; we consider three of those limitations here. First, paired normal tissues could not be obtained for the specimens examined in our study. Thus, it remains possible that some of the mutations classified as somatic events in the present study may in fact be germline mutations in this Japanese

**Table 5.** Clinically relevant or potentially clinically relevant somatic mutations except for *TP53* mutation and clinical features in our cohort.

| Gene | Exon | Mutation | Variant pattern | VF | COSMIC ID | ClinVar annotation | CIViC evidence level | OncoKB Evidence level | Clinical relevance | Histology | Sex | Age (years) | Smoking status | p-stage |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AKT1 | 4 | E17K | missense | 0.32817 | 33765 | Pathogenic/Likely pathogenic | B | 3A | Drug response to AZD5363 (capivasertib, Akt-i) | w/Ad | F | 56 | never | IIIA |
| EGFR | 19 | E746_A750 Del | indel | 0.73915 | 6223 | Drug response | A | 1 | Longer PFS by EGFR-TKI in NSCLC | w/Ad | F | 63 | never | IIB |
| FBXW7 | 10 | R505G | missense | 0.30651 | 99604 | Likely pathogenic | NA | NA (Likely oncogenic) | NA | w/Ad | M | 72 | ever | IIIA |
| KRAS | 2 | G12D | missense | 0.3599 | 521 | Pathogenic | B | R1, 3A, 4 | Poor PFS and OS by anti-EGFR Ab in CRC<br>Drug response to MEK-i and CDK 4/6-i in CRC | SCLC | F | 73 | never | IIIA |
| KRAS | 3 | Q61H | missense | 0.18395 | 1135364 | Pathogenic/Likely pathogenic | B | R1, 3A, 4 | Poor PFS and OS by anti-EGFR Ab in CRC<br>Drug response to MEK-i and CDK 4/6-i in CRC | w/La | M | 61 | unknown | IA |

VF, variant frequency; Age, age at diagnosis; p-stage, pathological stage; Akt-i, Akt inhibitor; w/Ad, combined with adenocarcinoma; EGFR, epidermal growth factor receptor; indel, insertion and deletion; PFS, progression free survival; TKI, tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; FBXW7, F-box and WD repeat domain containing 7; NA, not available; OS, overall survival; CRC, colorectal cancer; Ab, antibody; MEK-i, MEK inhibitor; CDK 4/6-i, cyclin dependent kinase 4/6 inhibitor; SCLC, small cell lung cancer; w/La, combined with large cell carcinoma.

cohort, although we screened for such lesions using various web databases.

Second, our TES system does not cover all exons of each of the 26 template genes, which might have compromised our ability to assess less-frequent, non-canonical gene mutations.

Third, ours was a retrospective observational study recruiting a heterogeneous population with a variety of treatments before and after surgery and representing a limited number of patients. Thus, it is possible that we would not have been able to detect precise associations between gene mutation profiles and survival.

In conclusion, TES of cancer-related genes by NGS and comparison with web databases, as used in this study, permitted us to identify several meaningful gene mutations that were predicted to alter drug response and survival in SCLC. However, our analysis surveyed a limited number of clinically relevant genes. Thus, an investigation of a larger gene panel in the TES system is recommended. The OncoGuide NCC Oncopanel System (Sysmex Corporation, Kobe, Japan), a panel that covers 114 genes, and FoundationOne (Foundation Medicine Inc. Cambridge, MA, USA), a panel that covers 324 genes, both received insurance coverage for use in cancer genome profiling in Japan in June 2019. Accumulation of gene alteration data and correlation with various clinical variables by these panels for research use may facilitate exploration of drug targets and promote reverse-translational research, including that on *TP53* mutations. These efforts are expected eventually to assist the development of precision treatments for patients with SCLC.

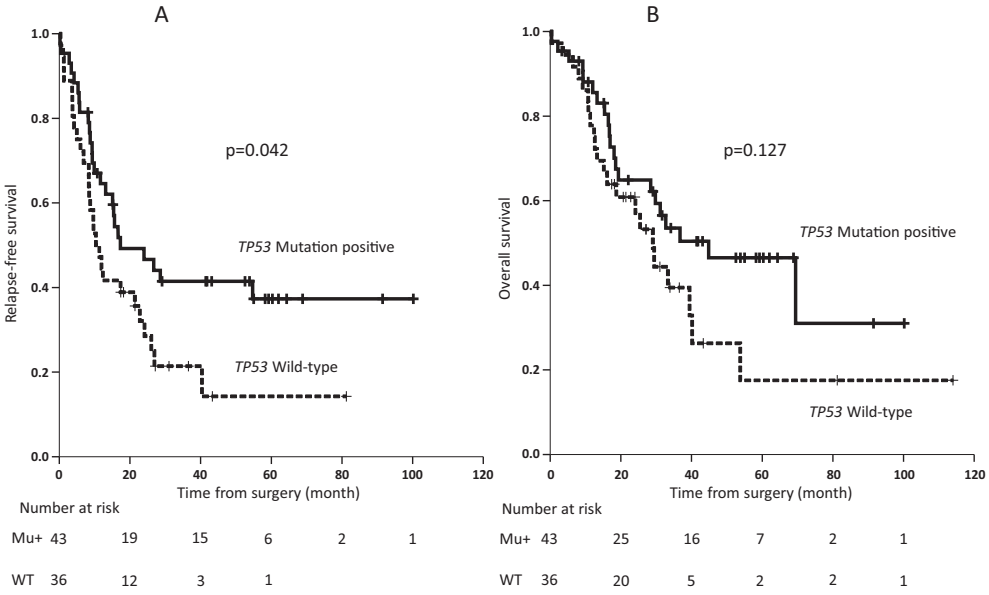


**Figure 3.** Kaplan–Meier curves of relapse-free survival (RFS) (A) and overall survival (OS) (B). Bold lines denote patients with nonsynonymous somatic *TP53* mutations, and dashed lines indicate those without such mutations. Vertical bars indicate the censored cases at the data cutoff point. Mu+, mutation positive; WT, wild type.

**Table 6.** Univariate analysis of the association between clinical variables and RFS.

| Variables | HR | 95%CI | *p* value |
|---|---|---|---|
| Age <70 years | 1.24 | 0.72–2.13 | 0.437 |
| Female | 1.00 | 0.55–1.83 | 0.990 |
| Never-smoker | 1.01 | 0.40–2.54 | 0.989 |
| ECOG PS: 0 | 0.80 | 0.44–1.45 | 0.464 |
| Combined SCLC | 1.77 | 0.94–3.33 | 0.078 |
| Without history or presence of other types of cancer | 0.93 | 0.51–1.67 | 0.799 |
| Without IP complication | 0.95 | 0.41–2.23 | 0.909 |
| Serum level of LDH < ULN | 0.63 | 0.35–1.12 | 0.113 |
| VATS approach | 0.60 | 0.35–1.03 | 0.064 |
| Lobectomy | 0.45 | 0.26–0.78 | 0.005 |
| p-stage IA | 0.40 | 0.22–0.74 | 0.003 |
| Adjuvant chemotherapy | 0.55 | 0.31–0.96 | 0.036 |
| PCI | 0.34 | 0.08–1.39 | 0.133 |
| *TP53* mutation | 0.57 | 0.33–0.99 | 0.044 |

RFS, relapse-free survival; HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; SCLC, small-cell lung cancer; IP, interstitial pneumonitis; LDH, lactate dehydrogenase; ULN, upper limit of normal range; VATS, video-assisted thoracoscopic surgery; p-stage, pathological stage; PCI, prophylactic cranial irradiation. Cox proportional hazard model analysis was used to obtain p values.

**Table 7.** Multivariate analysis of the association between clinical variables and RFS.

| Variables | HR | 95%CI | *p* value |
|---|---|---|---|
| Lobectomy | 0.46 | 0.26–0.80 | 0.007 |
| p-stage IA | 0.36 | 0.19–0.66 | 0.001 |
| Adjuvant chemotherapy | 0.55 | 0.32–0.97 | 0.038 |
| *TP53* mutation | 0.51 | 0.29–0.89 | 0.019 |

RFS, relapse-free survival; HR, hazard ratio; CI, confidence interval; p-stage, pathological stage; Cox proportional hazard model analysis was used to obtain p values.

**Table 8.** Univariate analysis of the association between clinical variables and OS.

| Variables | HR | 95% CI | *p* value |
|---|---|---|---|
| Age <70 years | 1.01 | 0.55–1.84 | 0.983 |
| Female | 0.80 | 0.40–1.59 | 0.523 |
| Never-smoker | 0.99 | 0.35–2.79 | 0.987 |
| ECOG PS: 0 | 0.86 | 0.44–1.68 | 0.661 |
| Combined SCLC | 1.71 | 0.86–3.42 | 0.129 |
| Without history or presence of other types of cancer | 0.69 | 0.37–1.31 | 0.258 |
| Without IP complication | 0.68 | 0.29–1.62 | 0.383 |
| Serum level of LDH < ULN | 1.01 | 0.51–2.00 | 0.986 |
| VATS approach | 0.77 | 0.42–1.40 | 0.386 |
| Lobectomy | 0.49 | 0.27–0.89 | 0.020 |
| p-stage IA | 0.41 | 0.21–0.82 | 0.011 |
| Adjuvant chemotherapy | 0.61 | 0.33–1.14 | 0.119 |
| PCI | 0.48 | 0.12–2.00 | 0.313 |
| *TP53* mutation | 0.63 | 0.34–1.15 | 0.130 |

OS, overall survival; HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; SCLC, small-cell lung cancer; IP, interstitial pneumonitis; LDH, lactate dehydrogenase; ULN, upper limit of normal range; VATS, video-assisted thoracoscopic surgery; p-stage, pathological stage; PCI, prophylactic cranial irradiation. Cox proportional hazard model analysis was used to obtain p values.

**Table 9.** Multivariate analysis of the association between clinical variables and OS.

| Variables | HR | 95% CI | *p* value |
|---|---|---|---|
| Lobectomy | 0.46 | 0.24–0.85 | 0.013 |
| p-stage IA | 0.42 | 0.21–0.85 | 0.015 |
| Adjuvant chemotherapy | 0.62 | 0.33–1.16 | 0.135 |
| *TP53* mutation | 0.62 | 0.33–1.15 | 0.126 |

OS, overall survival; HR, hazard ratio; CI, confidence interval; p-stage, pathological stage. Cox proportional hazard model analysis was used to obtain p values.

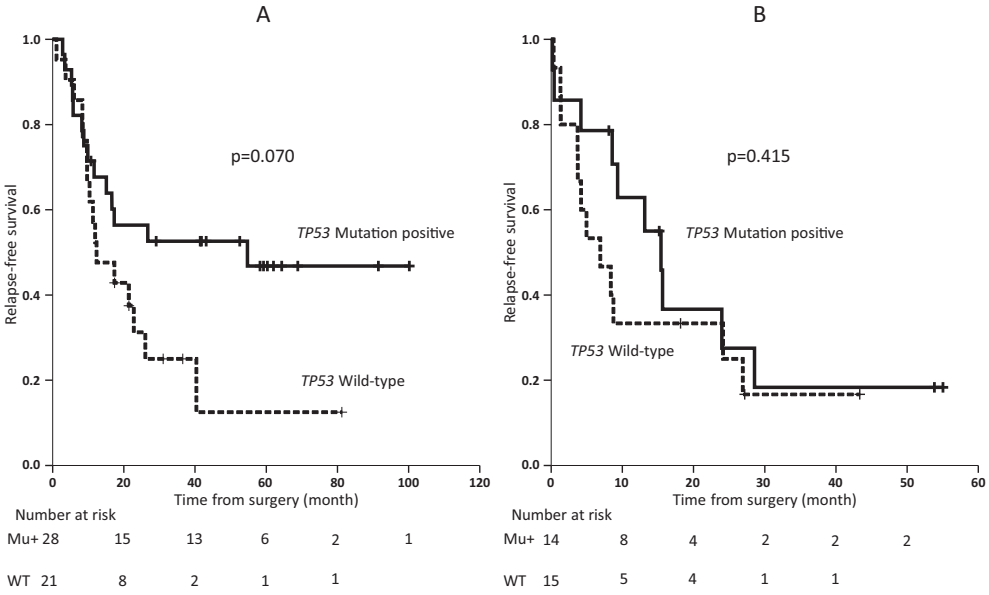


**Figure 4.** Kaplan–Meier curves of relapse-free survival (RFS) in patients who underwent adjuvant chemotherapy (A) and those who did not (B). Bold lines denote patients with nonsynonymous somatic *TP53* mutations, and dashed lines indicate those without such mutations. Vertical bars indicate the censored cases at the data cutoff point. Mu+, mutation positive; WT, wild type.

## Declarations

### *Author contribution statement*

H. Yokouchi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

H. Nishihara: Conceived and designed the experiments; Analyzed and interpreted the data.

T. Harada: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

S. Yamazaki, H. Kikuchi, H. Uramoto, M. Harada, K. Akie, F. Sugaya, Y. Fujita, K. Takamura, T. Kojima, M. Higuchi, O. Honjo, Y. Minami, and N. Watanabe: Contributed reagents, materials, analysis tools or data.

S. Oizumi, F. Tanaka, H. Suzuki, H. Dosaka-Akita, H. Isobe, and M. Nishimura: Analyzed and interpreted the data.

### *Funding statement*

This work was supported by research funding the Department of Translational Pathology, Hokkaido University Graduate School of Medicine; the Center for Respiratory Diseases, JCHO Hokkaido Hospital; and the Department of Pulmonary Medicine, Fukushima Medical University.

### *Competing interest statement*

The authors declare no conflict of interest.

### *Additional information*

The clinical trial described in this paper was registered at UMIN-CTR Clinical Trial under the registration number UMIN000010117.

## Acknowledgements

The authors thank all the patients and families who provided written consent and participated in this study. The authors also thank Dr. Yasuhiro Chikaishi (University of Occupational Environmental Health) for participating and collecting data for use in this study. We thank Ms. Hiromi Mohri (Department of Cancer Pathology, Hokkaido University Graduate School of Medicine) for technical assistance, data collection, and data interpretation of NGS. The authors also express their great appreciation to the late Dr. Mitsuru Munakata, formerly a professor emeritus at Fukushima Medical University School of Medicine, for his intellectual support of this study.

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