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Original Article

Relationship Between Mutation Profile Detected by Next-generation Sequencing and Histopathological Parameters in Lung Squamous Cell Carcinoma

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Abstract

Background and objectives: Lung Squamous cell carcinoma (LSCC) represents the second most common non-small cell lung cancer. Although studies identified adenocarcinoma-like driver mutations in LSCC using next-generation sequencing (NGS), the disease is challenging to treat due to the limited number of detectable mutations for targeted drug therapy. This study aimed to evaluate the mutation profiles of LSCC detected by NGS to assess the relationships between different driver mutations and clinicopathological parameters. ***Methods:*** NGS with a panel of 72 cancer-related genes was used to evaluate the driver mutation profiles of 41 lung resection specimens from patients with LSCC at the Molecular Pathology Laboratory of Aydın Adnan Menderes University in Türkiye. Clinical and histopathological features were recorded for analysis. ***Results:*** Detection of 94 mutations in 23 genes in DNA extracted from the tissue samples of 36 patients revealed that the most prevalent mutations were *TP53* (30.85%), *NF1* (20.20%), *PTEN* (11.70%), *PIK3CA* (5.31%), *FBXW7* (4.25%), *KRAS* (3.20%), respectively. We identified statistically significant relationships between *PIK3CA* and lower mean age (p = 0.007) and between *PTEN* and mild inflammatory reaction (p = 0.004). *PTEN* was associated with central localization (p = 0.13), *NF1* with visceral pleural involvement (p = 0.09), and *PIK3CA* with severe inflammatory reaction (p = 0.053), as well as with advanced pathological T stage (p = 0.09) and pathological N stage (p = 0.057) according to the TNM staging system. ***Conclusions:*** Our study highlights the importance of assessing mutation profiles in LSCC patients to identify driver mutations as potential therapeutic targets. Certain histopathological features are associated with these mutations, serving as indicators for treatment and follow-up decisions.

Keywords: Non-small cell lung cancer; Squamous cell carcinoma; Driver mutation; Next generation sequencing; p53; *PIK3CA*.

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Introduction

Lung squamous cell carcinoma (LSCC) is the second most prevalent tumor group among non-small cell lung cancers (NSCLCs).[1] There are significant differences between LSCC and the most common lung adenocarcinoma (LADC). These differences include the fact that LSCC originates from basal bronchiolar cells, as well as variations in tumor growth patterns, spread, metastatic behavior, and biological and molecular characteristics.[2] Studies have shown an association between smoking and LSCC development, with a high prevalence observed among middle-aged and elderly male patients, particularly those with a prolonged history of smoking.[1,2]

Despite advances in cancer biology, the prognosis for LSCC remains dismal. In recent decades, researchers have made significant improvements in the treatment of LADC through the identification of targetable driver mutations that underlie oncogenesis. The most prevalent therapeutic targets for LADC include mutations in the epidermal growth factor receptor (EGFR) and B-Raf proto-oncogene, serine/threonine kinase (BRAF) mutations, as well as rearrangements in ALK receptor tyrosine kinase (ALK), and ROS proto-oncogene 1, receptor tyrosine kinase (ROS1). Mutations such as those in the MET proto-oncogene, receptor tyrosine kinase (MET), RET proto-oncogene (RET), neurotrophic tyrosine receptor kinase (NTRK), and erb-b2 receptor tyrosine kinase 2 (HER2) show varying degrees of response in clinical trials.[3] Molecular studies have indicated that driver mutations are present in LSCCs at rates similar to or even higher than those in LADC.[2,4] The key difference is that mutations frequently detected in LADC are less common in LSCC. The identification

of biomarkers associated with tyrosine kinase inhibitor-based treatments and immunotherapy facilitates their utilization in late-stage LSCC patients.[1] Nevertheless, the prognosis remains poor, with a low five-year survival rate.[5] Currently, there are few mutations in LSCC for which targeted therapies are available; therefore, it remains a challenging disease to treat. To enhance the likelihood of successful treatment, it is imperative to comprehensively profile the gene mutations in LSCC and identify clinically significant mutations.[3]

Histological types of cancers are influenced by various risk factors, including molecular and environmental characteristics, resulting in unique mutation profiles. For this reason, revealing the molecular profile of individual tumors enables a more comprehensive understanding of the different molecular mechanisms that regulate cancer progression and the discovery of potential therapeutic targets.[6] Next-generation sequencing (NGS) is a highly effective technique for investigating the mutation profile of cancer, exhibiting high sensitivity and specificity across many cancer types. The advent of NGS has profoundly impacted the landscape of diagnosis and treatment processes, especially in lung cancer.[3] In the era of personalized medicine, NGS plays a pivotal role in identifying the most effective treatment options for each patient. Following the widespread adoption of NGS technology, the mutational structure of LSCC has also been a subject of investigation.[1,3,7] Numerous studies have identified various mutated genes, including *TP53*, *PIK3CA*, *CDKN2A*, *SOX2*, and *CCND1*, among others. Notably, mutations in *PIK3CA*, *HER2*, and *FGFR1* have demonstrated therapeutic efficacy in treating LSCC.[1,7]

Many studies in the literature have focused on molecular changes, therapeutic strategies, and prognoses in advanced-stage LSCC cases that are no longer candidates for surgery. These cases may benefit from targeted treatments and immunotherapies. However, there is a lack of research dedicated to the group of cases that includes resected early-stage patients, as surgery is the primary treatment for most of these cases, and they exhibit significantly longer survival times. The existing literature has not thoroughly investigated the prognosis of resected LSCC, as it constitutes a small percentage of NSCLC cases.[1] In this retrospective single-center study, we evaluated the relationship between different oncogenic driver mutations and clinical and histopathological parameters by analyzing the mutation profile detected through NGS in resected LSCC cases. In conjunction with the histopathological factors evaluated in resection materials, we aimed to obtain actionable results and potential biomarkers to predict the prognosis of LSCC cases with resectable tumors. This effort also sought to evaluate treatment and follow-up situations, thus enhancing our understanding of the various molecular mechanisms that regulate cancer progression.

Materials and methods

Patient selection

The study included 41 patients diagnosed with LSCC who underwent surgical resection, with NGS performed on tumor tissue. Cases were excluded if surgical resection was not performed, the excision material was not examined in the laboratory, or NGS was conducted on tru-cut biopsies. The study was conducted from January 2019 to June 2024 using the NGS system at the Molecular Pathology Laboratory of the Department of Medical Pathology at Aydın Adnan Menderes University Faculty of Medicine in Türkiye.

Histopathological parameters documented in the pathology reports of the resection materials were systematically recorded, including material type, tumor localization, laterality, tumor size, bronchial surgical margin, visceral pleural involvement, inflammatory reaction, presence of necrosis, lymphovascular invasion, lymph node involvement, and pTNM staging (as per the American Joint Committee on Cancer [AJCC] 8th Edition).[8] Additionally, patient demographics such as age, gender, smoking history, mutation status, genes with mutations, complementary DNA, protein changes, and exon information (72 cancer-related genes; 4,149 primary analysis results) were recorded (see Table 1). Histopathological parameters were organized according to the 2021 World Health Organization Classification of Lung Tumors,[9] which is utilized in lung resection materials, as well as the pTNM classification from the AJCC 8th Edition,[8] which serves as the staging system. The College of American Pathologists Cancer Protocols guide was also followed to ensure that all pathology reports contained the necessary data for patient treatment and follow-up.

Table 1. Lung cancer panel study with next-generation sequencing (DHS-005Z-12 & DHS-005Z-96)

AKT1	*FBXO7*	*MGA*	*PTPRD*
ALK	*FBXW7*	*MLH1*	*RARB*
AMER1	*FGFR1*	*MUC16*	*RASSF1*
APC	*FGFR2*	*MYC*	*RB1*
ARID1A	*FGFR3*	*NF1*	*RBM10*
ATM	*FHIT*	*NFE2L2*	*RET*
BAI3	*GRM8*	*NOTCH1*	*RIT1*
BAP1	*HRAS*	*NRAS*	*ROS1*
BRAF	*JAK2*	*NTRK1*	*RUNX1T1*
CDKN2A	*KDR*	*NTRK2*	*SETD2*
CDKN2B	*KEAP1*	*NTRK3*	*SMAD4*
CREBBP	*KIT*	*PDGFRA*	*SMARCA4*
CTNNB1	*KMT2D*	*PIK3CA*	*SOX2*
DDR2	*KRAS*	*PIK3CG*	*SKT11*
EGFR	*LRP1B*	*PIK3R1*	*TNFAIP3*
EPHAS	*MAP2K1*	*PIK3R2*	*TP53*
ERBB2	*MDM2*	*PKHD1*	*TSC1*
ERBB4	*MET*	*PTEN*	*U2AF1*

NGS method

The materials from the patients included in the study were tested using the Illumina Platform NGS system (Qiagen, Hilden, Germany). The lung cancer panel of the NGS system recorded 72 cancer-associated genes and 4,149 variant analysis results (Table 1). Tumor cellularity was the primary inclusion criterion. The NGS cut-off value was set at 5%.[10] Patients with low DNA quality, inadequate library concentration, median read coverage, and insufficient homogeneity were excluded from the study. Ultimately, 41 patients met the study's inclusion criteria.

The NGS workflow is outlined below. Initially, genomic tumor DNA was isolated from tissue samples. Subsequently, DNA that met the necessary quality and quantity criteria underwent target region enrichment. Finally, researchers constructed and sequenced the study libraries. The resulting

data underwent variant analysis and bioinformatic interpretation, taking into account the patients' clinical history.

DNA extraction

Tumor areas were marked by a pathologist on hematoxylin-eosin slides, and 10 µm thick sections were cut. These sections were placed in a tube containing proteinase K and lysis buffer at 56°C for 12–24 h. After lysis, genomic DNA was extracted using the Qiagen formalin-fixed paraffin-embedded DNA Tissue Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and purity were measured using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and a NanoDrop spectrophotometer. An average of 100–150 ng DNA was considered sufficient for the study, allowing the workflow to continue with these samples.

Library preparation and sequencing

Following DNA isolation from formalin-fixed, paraffin-embedded tissues, the subsequent steps were executed. Initially, the isolated DNA samples were fragmented using an enzymatic method. Adapter ligation was then performed on the fragmented DNA using the NEBNext Ultra II DNA Library Prep Kit (New England Biolab, Ipswich, MA, USA). After ligation, the library fragments were amplified by polymerase chain reaction (Labcycler, Sensoquest GmbH, Göttinger, Germany) to enrich the target region. The quantity and size of the prepared libraries were assessed using a Qubit fluorometer and an Agilent 2100 Bioanalyzer. The target-enriched library was subsequently subjected to sequencing to detect mutations and co-mutations using the MiniSEQ High Output Reagent Cartridge (Illumina, Inc., San Diego, California, USA) on MiniSEQ NGS platforms (MiniSEQ, MN00 676, Illumina, Hayward, CA, USA) and the Lung Cancer NGS panel (DHS-005Z-12, Qiagen, Strasse, Hilden, Germany; Table 1) in accordance with the manufacturer's instructions.

Mutation analysis and bioinformatics

In the NGS system, mutations detected in cancer-associated genes were categorized into four tiers (Tiers I-IV) according to the Association for Molecular Pathology/American Society of Clinical Oncology/College of American Pathologists guidelines, based on their relevance to cancer diagnosis, prognosis and/or therapy.[11] In this study, only pathogenic (Tier I, Stage IA, IB) and likely pathogenic (Tier II, Stage IIC, IID) mutations were evaluated, considering validated therapeutic, prognostic, and diagnostic variables. These mutations predict response/resistance to Food and Drug Administration or European Medicines Agency approved treatments based on drug labeling, diagnostic professional guidelines, or robust clinical trials.

To assess data quality, raw data were obtained in FASTQ format and subjected to quality control using FASTQC software v.1.0.0. Cleaned sequences were aligned to the reference human genome (GRCh38) using Burrows-Wheeler Aligner software v.1.1.5. Data analysis was performed using MiniSEQ software v.2.3, and quality indicators were evaluated using QCI analysis (Qiagen). Variants were imported into the Qiagen Clinical Insight interpretation web interface (QIAGEN Clinical Insight Interpret 9.0.0.20220826) for data interpretation. Variant selection was performed using Clinical Insight and Ingenuity software v.8.1.202021 (Qiagen). Data were visually inspected using IGV 2.8.2 software. Selected mutations were analyzed using bioinformatics tools (QIAGEN Clinical Insight Interpret 9.1.0.20230224) to assess their impact on diagnostic confirmation, clinical data, and treatment protocols. Variations in cases were identified using the Qiagen Reporter and Qiagen Clinical Insight Browser platforms. For each identified variant, a report was generated per sample, summarizing the findings and providing a direct link to the data source with recommended treatment. The detection limit for this panel was set at 5%. The NGS testing process took an average of one week.

Statistical analysis

All statistical analyzes were performed with SPSS version 26.0 software (SPSS, Chicago, IL, USA). In descriptive statistics for the variables, categorical variables were expressed as the number of cases and percentages (%), while discrete numerical variables were presented as mean ± standard deviation. Pearson's χ^2 test was used in the analyses of categorical data unless otherwise stated. In 2×2 cross-tabulations, the χ^2 test with continuity correction was applied when the expected frequency was between 5 and 25. If the expected frequency was below 5 in at least 25% of the cells, Fisher's exact probability test was utilized. Measurement data were evaluated using Student's *t*-test and Mann–Whitney's U tests, according to their distribution. A *p*-value of <0.05 results was considered statistically significant.

Results

The mean age of the 41 cases included in the study was 68.4 years (age range: 53–83 years, standard deviation = 7.2). There was a male predominance, with only three female patients. Table 2 provides a synopsis of the clinicopathological characteristics by gender.

Evaluation of mutation profile detected by NGS

The study identified 94 mutations (61 pathogenic and 33 likely pathogenic) in 36 of the 41 cases examined. The detailed examination revealed 75 different variants (49 pathogenic and 26 likely pathogenic) across 23 different genes. The distribution of all detected mutations revealed that *TP53* was the most frequent mutation (30.85%, 29/94), followed by *NF1* (20.20%, 19/94), *PTEN* (11.70%, 11/94), *PIK3CA* (5.31%, 5/94), *FBXW7* (4.25%, 4/94), and *KRAS* (3.20%, 3/94). Figure 1 shows the distribution of all detected mutations.

Relationship of mutations with histopathological parameters

Analysis of the relationship between the six most frequently detected mutations and clinical and histopathological parameters revealed a statistically significantly lower mean age in those with the *PIK3CA* mutation ($p = 0.007$). Although cases with this mutation exhibited a higher severity of inflammatory reaction ($p = 0.053$), advanced pathological T stage (pT; $p = 0.09$), pathological N stage (pN; $p = 0.057$), and advanced pT clinical stage ($p = 0.053$) compared to the general population, these results were not statistically significant. The presence of mild inflammatory reaction in patients carrying the *PTEN* mutation was statistically significant ($p = 0.004$). While not statistically significant, the frequency of central localization in patients with *PTEN* mutation was 77.7%, compared to 50% in patients without this mutation ($p = 0.13$). The rate of visceral pleural involvement in patients with *NF1* mutation was 23%, while it was 50% in patients without it, indicating a lower rate of visceral pleural involvement associated with the *NF1* mutation ($p = 0.09$; Table 3).

Concomitant mutations and their relationship with histopathological parameters

Mutations were detected in 36 patients: 11 had a single mu-

Table 2. Clinical and histopathological characteristics of patients according to gender

	Male (%)	Female (%)	N (%)	*p*-value
Mean age (years)	69 ± 7.1	62 ± 4.5	68,4 ± 7.2	0.07
Smoking history				0.67
Present	33 (91.7%)	3 (8.3%)	36 (100%)	
Absent	5 (100%)	0 (0%)	5 (100%)	
Type of resection				0.97
Segmentectomy	5 (100%)	0 (0%)	5 (100%)	
Lobectomy	27 (96.4%)	1 (3.6%)	28 (100%)	
Pneumonectomy	6 (75%)	2 (25%)	8 (100%)	
Laterality				0.57
Right lung	22 (95.7%)	1 (4.3%)	23 (100%)	
Left lung	16 (88.9%)	2 (11.1%)	18 (100%)	
Localization				0.24
Central localization	20 (87%)	3 (13%)	23 (100%)	
Peripheral localization	18 (100%)	0 (0%)	18 (100%)	
Tumor size	4 ± 2.6	4.6 ± 4.2	4 ± 2.7	0.91
Bronchial surgical margin				0.92
Negative	37 (92.5%)	3 (7.5%)	40 (100%)	
Positive	1 (100%)	0 (0%)	1 (100%)	
Border of visceral pleura				0.51
PL0	21 (87.5%)	3 (12.5%)	24 (100%)	
PL1	4 (100%)	0 (0%)	4 (100%)	
PL2	3 (100%)	0 (0%)	3 (100%)	
PL3	10 (100%)	0 (0%)	10 (100%)	
Inflammatory reaction				0.70
Mild	19 (90.5%)	2 (9.5%)	21 (100%)	
Moderate	12 (92.3%)	1 (7.7%)	13 (100%)	
Severe	7 (100%)	0 (0%)	7 (100%)	
Lymphovascular invasion				0.67
Present	33 (91.7%)	3 (8.3%)	36 (100%)	
Absent	5 (100%)	0 (0%)	5 (100%)	
Presence of necrosis				0.43
Absent	8 (88.9%)	1 (11.1%)	9 (100%)	
Focal	16 (88.9%)	2 (11.1%)	18 (100%)	
Diffuse	14 (100%)	0 (0%)	14 (100%)	
Lymph node involvement				0.43
Present	19 (86.4%)	3 (13.6%)	22 (100%)	
Absent	18 (100%)	0 (0%)	18 (100%)	
Unknown	1 (100%)	0 (0%)	1 (100%)	
pT stage				0.12
pT1	10 (90.9%)	1 (9.1%)	11 (100%)	
pT2	14 (100%)	0 (0%)	14 (100%)	
pT3	8 (100%)	0 (0%)	8 (100%)	

(continued)

Table 2. *(continued)*

	Male (%)	Female (%)	N (%)	*p*-value
pT4	6 (75%)	2 (25%)	8 (100%)	
pN stage				0.16
pN0	18 (100%)	0 (0%)	18 (100%)	
pN1	13 (81.3%)	3 (18.8%)	16 (100%)	
pN2	6 (100%)	0 (0%)	6 (100%)	
Unknown	1 (100%)	0 (0%)	1 (100%)	
Mutation status				0.67
Present	33 (91.7%)	3 (8.3%)	36 (100%)	
Absent	5 (100%)	0 (0%)	5 (100%)	
Concomitant mutation				0.69
Without any mutation	5 (100%)	0 (0%)	5 (100%)	
Single gene mutation	10 (90.9%)	1 (9.1%)	11 (100%)	
Two gene mutations	8 (100%)	0 (0%)	8 (100%)	
Three or more gene mutations	15 (88.2%)	2 (11.8%)	17 (100%)	
Total	38 (92.7%)	3 (7.3%)	41 (100%)	

tation, eight had two mutations, and 17 had three or more. Among those with a single mutation, five had *TP53*, two had *PIK3CA*, and one each had mutations in *FGFR1*, *AK2*, *KRAS*, and *SMARCA4*. The most prevalent double concomitant mutations were *TP53-NF1* in 10 patients, *TP53-PTEN* in eight, *NF1-PTEN* in five, *TP53-FBXW7* in four, and *NF1-FBXW7* in three patients. The most prevalent triple concomitant mutations were *NF1-PTEN-TP53* in four patients and *FBXW7* + *NF1* + *TP53* in three (Fig. 2).

Our examination of the relationship between concomitant mutations and histopathological parameters revealed that the *TP53-NF1* mutation association was linked to lower rates of visceral pleural involvement, although this result was not statistically significant (p = 0.11). The rate of visceral pleura involvement was 20% in cases with these two mutations, compared to 48.4% in cases without them (p = 0.11). A similar relationship was observed between *TP53-NF1* mutation coexistence and the incidence of necrosis (p = 0.06). Necrosis was present in all cases with these two mutations, with 60% exhibiting extensive areas of necrosis. In contrast, in the absence of this mutation association, these rates were 71% and 25.8%, respectively (p = 0.06). A statistically significant difference was identified between the association of the *TP53-PTEN* mutation and low inflammatory response; all patients in the low inflammatory response group had this mutation, while this rate was 39.4% in patients without this mutation pair (p = 0.02).

Discussion

LSCC, accounting for 30% of NSCLC cases, is often diag-

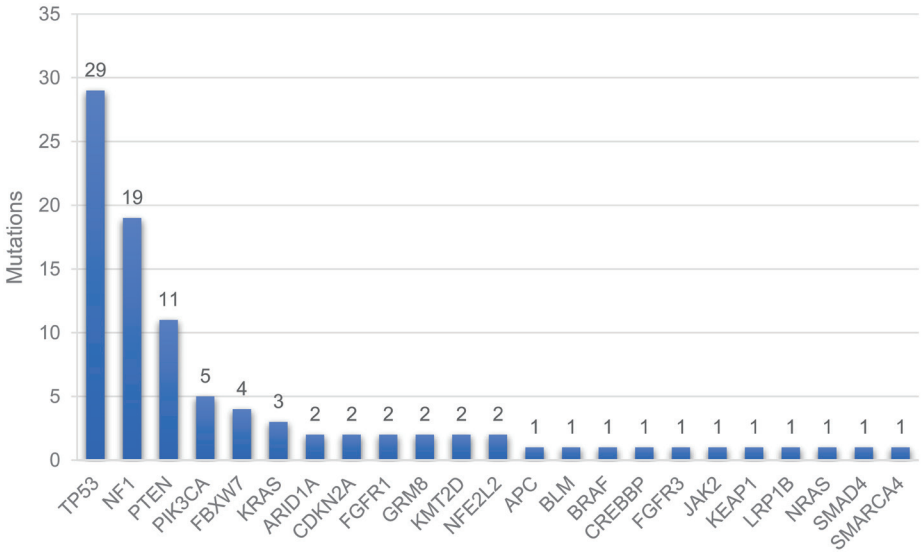

Fig. 1. Distribution of all mutations detected.

Table 3. Distribution of clinical and histopathological parameters in the six most frequently detected mutations

	TP53	***NF1***	***PTEN***	***PIK3CA***	***FBXW7***	***KRAS***
Mean age (years)	68.8 ± 1.3, p = 0.39	68.9 ± 1.5, p = 0.21	67 ± 2.5, p = 0.57	61.6 ± 4.3, p = 0.007	69.4 ± 3.1, p = 0.73	68 ± 4.9, p = 0.42
Gender	p = 0.73	p = 0.69	p = 0.11	p = 0.79	p = 0.67	p = 0.79
Female	2 (66.6%)	1 (33.3%)	2 (66.7%)	0 (0%)	0 (0%)	0 (0%)
Male	25 (65.8%)	12 (31.6%)	7 (18.4%)	3 (7.9%)	5 (13.2%)	3 (7.9%)
Smoking history	p = 0.2	p = 0.48	p = 0.7	p = 0.67	p = 0.5	p = 0.67
Present	25 (69.4%)	12 (33.3%)	8 (22.2%)	3 (8.3%)	5 (13.9%)	3 (8.3%)
Absent	2 (40%)	1 (20%)	1 (20%)	0 (0%)	0 (0%)	0 (0%)
Type of resection	p = 0.21	p = 0.8	p = 0.09	p = 0.47	p = 0.37	p = 0.47
Segmentectomy	16 (57.1%)	9 (32.1%)	4 (13.4%)	3 (10.7%)	3 (10.7%)	3 (10.7%)
Lobectomy	7 (87.5%)	3 (37.5%)	4 (50%)	0 (0%)	2 (25%)	0 (0%)
Pneumonectomy	4 (80%)	1 (20%)	1 (20%)	0 (0%)	0 (0%)	0 (0%)
Laterality	p = 0.4	p = 0.55	p = 0.42	p = 0.4	p = 0.38	p = 0.16
Rigt lung	16 (69.6%)	7 (30.4%)	4 (17.4%)	1 (4.3%)	2 (8.7%)	3 (13%)
Left lung	11 (61.1%)	6 (33.3%)	5 (27.8%)	2 (11.1%)	3 (16.7%)	0 (0%)
Localization	p = 0.33	p = 0.55	p = 0.13	p = 0.4	p = 0.61	p = 0.59
Central localization	13 (72.2%)	6 (33.3%)	2 (11.1%)	2 (11.1%)	2 (11.1%)	1 (5.6%)
Peripheral localization	14 (60.9%)	7 (30.4%)	7 (30.4%)	1 (4.3%)	3 (13%)	2 (8.7%)
Tumor size	3.7 ± 0.3, p = 0.36	3.9 ± 0.6, p = 0.5	4.1 ± 0.5, p = 0.8	5.2 ± 1.5, p = 0.75	3.8 ± 0.5, p = 0.74	3.6 ± 1.4, p = 0.14
Bronchial surgical margin	p = 0.65	p = 0.31	p = 0.22	p = 0.92	p = 0.87	p = 0.77
Negative	26 (65%)	12 (30%)	8 (20%)	3 (7.5%)	5 (12.5%)	3 (7.5%)
Positive	1 (100%)	1 (100%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)
Border of visceral pleura	p = 0.48	p = 0.36	p = 0,13	p = 0.35	p = 0.41	p = 0.35
PL0	18 (75%)	10 (41.7%)	6 (25%)	1 (4.2%)	4 (16.7%)	1 (4.2%)
PL1	2 (50%)	1 (25%)	0 (0%)	0 (0%)	1 (25%)	0 (0%)
PL2	2 (66.6%)	0 (0%)	2 (66.6%)	0 (0%)	0 (0%)	0 (0%)
PL3	5 (50%)	2 (20%)	1 (10%)	2 (20%)	0 (0%)	2 (20%)
Visceral pleural involvement	p = 0.12	p = 0.09	p = 0.43	p = 0.37	p = 0.29	p = 0.37
Absent (PL0)	18 (75%)	10 (41.7%)	6 (25%)	1 (4.2%)	4 (16.7%)	1 (4.2%)
Present (PL1,2,3)	9 (52.9%)	3 (17.6%)	3 (17.6%)	2 (11.8%)	1 (5.9%)	2 (11.8%)
Inflammatory reaction	p = 0.36	p = 0.96	p = 0.004	p = 0.053	p = 0.83	p = 0.18
Mild	15 (71.4%)	7 (33.3%)	9 (42.9%)	1 (4.8%)	3 (14.3%)	0 (0%)
Moderate	9 (69.2%)	4 (30.8%)	0 (0%)	0 (0%)	1 (7.7%)	2 (15.4%)
Severe	3 (42.9%)	2 (28.6%)	0 (0%)	2 (28.6%)	1 (14.3%)	1 (14.3%)
Lymphovascular invasion	p = 0.2	p = 0.48	p = 0.26	p = 0.67	p = 0.49	p = 0.035
Present	25 (69.4%)	12 (33.3%)	9 (25%)	3 (8.3%)	4 (11.1%)	1 (2.8%)
Absent	2 (40%)	1 (20%)	0 (0%)	0 (0%)	1 (20%)	2 (40%)
Presence of necrosis	p = 0.22	p = 0.2	p = 0.99	p = 043	p = 0.3	p = 0.11
Focal	14 (77.8%)	4 (22.2%)	4 (22.2%)	2 (11.1%)	2 (11.1%)	0 (0%)
Diffuse	9 (64.3%)	7 (50%)	3 (21.4%)	0 (0%)	3 (21.4%)	1 (7.1%)
Absent	4 (44.4%)	2 (22.2%)	2 (22.2%)	1 (11.1%)	0 (0%)	2 (22.2%)
Lymph node involvement	p = 0.27	p = 0.57	p = 0.25	p = 0.24	p = 0.71	p = 0.7

(continued)

Table 3. *(continued)*

	TP53	***NF1***	***PTEN***	***PIK3CA***	***FBXW7***	***KRAS***
Present	16 (82.7%)	6 (27.3%)	7 (31.8%)	3 (13.6%)	2 (9.1%)	1 (4.5%)
Absent	11 (61.1%)	7 (38.9%)	2 (11.1%)	0 (0%)	3 (16.7%)	2 (11.1%)
Unknown	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
pT stage	p = 0.58	p = 0.42	p = 0.82	p = 0.09	p = 0.5	p = 0.14
pT1	7 (63.6%)	3 (27.3%)	2 (18.2%)	0 (0%)	1 (9.1%)	1 (9.1%)
pT2	11 (78.6%)	5 (35.7%)	4 (28.6%)	0 (0%)	3 (21.4%)	0 (0%)
pT 3	5 (62.5%)	4 (50%)	1 (12.5%)	2 (25%)	1 (12,5%)	2 (25%)
pT 4	4 (50%)	1 (12.5%)	2 (25%)	1 (12.5%)	0 (0%)	0 (0%)
pTNM Clinical stage	p = 0.24	p = 0.61	p = 0.5	p = 0.053	p = 0.34	p = 0.33
Early stage (1,2)	18 (72%)	8 (32%)	6 (24%)	0 (0%)	4 (16%)	1 (4%)
Advanced stage (3,4)	9 (56.3%)	5 (31.3%)	3 (18.8%)	3 (18.8%)	1 (6.3%)	2 (12.5%)
pN stage	p = 0.43	p = 0.67	p = 0.42	p = 0.057	p = 0.72	p = 0.81
pN0	11 (61.1%)	7 (38.9%)	2 (11.1%)	0 (0%)	3 (16.7%)	2 (11.1%)
pN1	12 (75%)	5 (31.3%)	5 (31.3%)	1 (6.3%)	2 (12.5%)	1 (6.3%)
pN2	4 (66.7%)	1 (16.7%)	2 (33.3%)	2 (33.3%)	0 (0%)	0 (0%)
Unknown	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	27	13	9	3	5	3

nosed at an advanced stage, exhibiting a high rate of metastasis and recurrence, which results in low survival rates and poor prognosis.[12,13] It is more prevalent among middle-aged and elderly male patients with a history of smoking.[13] Consistent with existing literature, our study found that the majority of cases (92.7%) were older male patients with a smoking history. LSCC is a tumor that develops with a high accumulation of multiple mutations, particularly the *TP53* gene. Tobacco mutagens are primarily responsible for *TP53* mutations, which trigger a multitude of molecular changes leading to cancer initiation and progression.[2]

Current treatment options for lung cancer include surgical resection, chemotherapy, radiotherapy, targeted therapy, and immunotherapy. Surgical resection is commonly utilized to treat early-stage lung cancer, while immunotherapy or targeted therapy combined with chemotherapy is the optimal

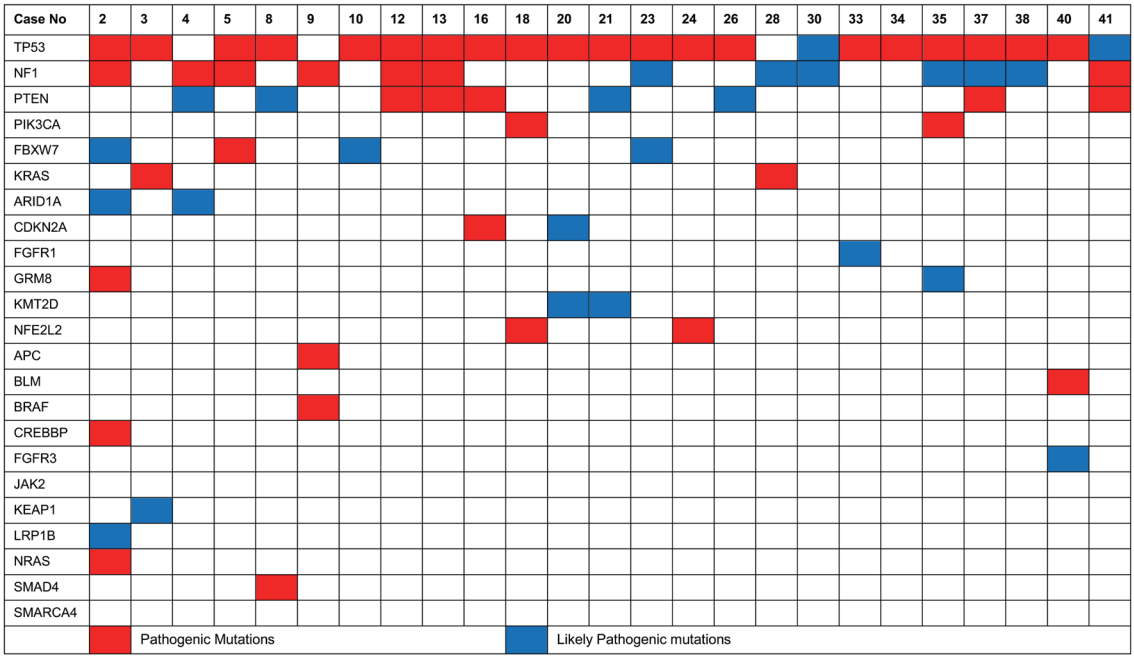

Fig. 2. Mutation distribution in patients with more than one mutation detected.

treatment option for advanced-stage patients whose tumors are not amenable to surgical removal.[13] Recent advancements in targeted therapies have identified effective molecular targets such as *EGFR* and *ALK*, which have proven effective in lung LADC but not in LSCC because of their distinct mutational profiles.[13,14] In contrast to LADC, targeted therapies offer limited benefits for LSCC patients.[12] Currently, the combination of chemotherapy and immune checkpoint inhibitors is the first-line treatment for LSCC in the clinic,[13] significantly improving treatment response and prolonging survival compared to chemotherapy alone in metastatic LSCC.[3,13] However, immunotherapy treatment is expensive and can cause serious side effects.[13] Additionally, it has been shown to be ineffective as a first-line treatment in LSCC patients with driver mutations.[3] Therefore, accurate identification of potential driver mutations in LSCC may enhance the selection and increased efficacy of first-line therapies.

Unlike LADC, LSCC remains a challenging disease to treat. The absence of Food and Drug Administration-approved targeted therapies for LSCC patients with gene mutations is a significant barrier.[12] While several studies have reported on genes and prognostic markers associated with LSCC, there has been limited systematic evaluation of specific molecular mechanisms in LSCC pathogenesis and progression, hindering early diagnosis and treatment.[13] A comprehensive understanding of the molecular mechanisms involved in LSCC formation and development is imperative for developing effective strategies for early diagnosis and subsequent clinical treatment. Consequently, there is an urgent need for novel biomarkers and drug therapies. NGS represents an important technological advancement, enabling the simultaneous detection of mutations in numerous genes. NGS-based studies have revealed that 13% of LSCC cases harbor at least one potentially actionable alteration,[15] while other studies suggest that up to 85% of LSCC cases contain a potentially actionable molecular alteration.[16,17] In our study, we detected mutations in one or more genes in approximately 88% of resected LSCC patients. Therefore, clinicians should avoid labeling a patient as "non-mutated" based solely on negative results for *EGFR* and *ALK* mutations or histopathology.

Mutation profiles in tumors may differ according to histological type, tumor microenvironment, and stage. Walter *et al*.[16] detected mutations in 13 different genes in LSCC, with the most common being *TP53* (38.5%), *PIK3CA* (21.9%), *KRAS* (12.5%), *EGFR* (12.5%), and *MET* (3.1%). Schwaederle *et al*.[18] identified common mutations in *TP53* (64.5%), *PIK3CA* (28.5%), *CDKN2A* (24.4%), *SOX2* (17.7%), and *CCND1* (15.8%) in LSCC patients (4–18). A study of 355 Chinese LSCC patients identified *TP53* (78%), *TTN* (68%), *SMD3* (39%), *UC16* (36%), and *RYR2* (36%) as the genes with the highest mutation frequency.[1] Another study found that *TP53* mutations were prevalent (90%, 93/103) among LSCC patients. Mutations were also found in *CDKN2* (20/103), *CCND1* (14/103), *PIK3CA* (40/103), and *FGFR1* (13/103).[6] In our study, the most frequently detected mutation was *TP53* (30.85%), followed by *NF1* (20.20%), *PTEN* (11.70%), *PIK3CA* (5.31%), *FBXW7* (4.25%), and *KRAS* (3.20%). While the prevalence of mutation type aligns with existing literature, discrepancies in other cases may be attributable to factors such as the population studied and the gene panel analyzed. Further research, particularly large-scale cohort studies, is necessary to validate the observed incidence rates.

TP53 mutations represent the most prevalent molecular alterations in LSCC, with their occurrence being associated with smoking. These mutations impair DNA damage repair, cellular apoptosis, and cell cycle regulation.[19] Although *TP53* is one of the most extensively studied prognostic markers with an established negative prognostic role, there is currently no proven targeted therapy available.[3] Some studies suggest that the *TP53* mutations in LSCC may confer resistance to platinum-based chemotherapy,[19,20] potentially due to alterations in DNA repair mechanisms required for treatment. Furthermore, immune features associated with *TP53* mutations, such as T cell infiltration and tumor mutational burden, may enhance responses to immunotherapy.[21] As a crucial tumor suppressor gene in humans, *TP53* is of considerable clinical importance for the development of targeted therapies. Mutations in *TP53* can lead to increased chromosomal instability in tumor cells, resulting in the amplification of oncogenes and the loss of other tumor suppressor genes.[19] Therefore, there is growing interest in exploring therapeutic strategies that target mutations in tumor suppressor genes, despite challenges such as drug resistance, inconsistent animal models, and limitations of single-agent *TP53* therapy, which have hindered the development of *TP53*-targeted drugs, with no drugs receiving approval thus far. Nevertheless, there is a compelling rationale for continued investigation into the biological properties of *TP53*.

Broad-panel molecular multigene testing has become increasingly important due to the increasing approval and development of targeted drugs for rare mutations.[22] However, our limited understanding of how molecular profiles differ between patients with early-stage disease and those with advanced-stage disease hinders the ability to tailor more precise therapeutic interventions. Additionally, although financial reimbursement for NGS testing is available in some countries, NGS is not universally accessible. Establishing distinct diagnostic patterns for different cases is imperative to accurately characterize the clinicopathological features of patients with specific mutations. This approach will conserve tumor tissue and reduce financial and temporal expenditures.

Most studies have focused on advanced-stage LSCC cases ineligible for surgery, primarily utilizing biopsy samples for diagnosis. The group of resectable cases, including early-stage patients, constitutes a small proportion of prognostic cases, leading to a paucity of research on this group.[16] In such studies, baseline characteristics of patients, including age, gender, smoking history, tumor histology, and clinical stage, have been correlated with NGS test results.[1,16] One study revealed that *PIK3CA* and *BRAF* mutations in LSCC were more prevalent in tumors diagnosed at earlier and more localized stages,[16] while another study found no significant difference in mutation frequency across stages I–III in the top mutated genes in LSCC.[1] There is currently no literature evaluating the relationship between detailed histopathological parameters from resection materials and mutation profiles. Our study found that cases with *PIK3CA* mutations were associated with younger age (p = 0.007), severe inflammatory reaction (p = 0.053), more advanced clinical stage (p = 0.053), and lymph node involvement (p = 0.057). Although *TP53* is the most prevalent mutation in LSCC is non-actionable, other prevalent potentially targetable alterations include phosphatidylinositol 3-kinase (PI3K) abnormalities.[3,4] A common mechanism of PI3K activation in cancers is the occurrence of mutations in the *PIK3CA* gene, with reported prevalence in LSCC ranging from 8% to 20%.[23] When mutations occur in *PIK3CA*, aberrant activation of the PI3K/ATK/mTOR signaling pathway promotes cancer cell proliferation and invasion. Moreover, *PIK3CA* mutations have been associated with chemotherapy resistance and poor

prognosis.[19] Our findings indicate that identifying this mutation in younger patients with advanced clinical stage and lymph node involvement corroborates established relationships. Additionally, the association with severe inflammatory response surrounding the tumor may serve as a predictor of response to immunotherapy in individuals carrying this mutation. Despite the efficacy limitations of immunotherapy due to resistance, the presence of both primary and secondary drug resistance remains prevalent. As a solution, identifying biomarkers that can predict the outcome and prognosis of immunotherapy remains crucial.[5] Currently, alpelisib, a drug targeting *PIK3CA*, has received approval for breast cancer treatment.[19] While LSCC has been the subject of many clinical trials, early-phase clinical trials have yielded negative results.[19] A more profound comprehension of PI3K's role in cancer and its microenvironment will facilitate the future development of more durable therapeutic responses with personalized PI3K-based therapies.

PTEN is a crucial negative regulator of the PI3K/AKT/mTOR signaling pathway, exhibiting a mutation rate of approximately 7–10% in LSCC, with the majority of these mutations occurring in smokers.[19,23] This may be related to smoking-induced downregulation of *PTEN* expression through immune-mediated mechanisms. Studies have shown that low PTEN protein expression levels are associated with reduced survival rates in lung cancer patients.[19,24] In our study, particularly in centrally located LSCC cases, *PTEN* mutation was associated with a low inflammatory response around the tumor. Partial loss of *PTEN* function may have a substantial impact on tumor formation and cancer progression.[23] Importantly, *PTEN* function is frequently not entirely lost in cancer, presenting an opportunity for reactivating a treatment strategy. It has been reported that systemic elevation of *PTEN* induces a tumor-suppressive metabolic state in transgenic mouse models. Lee *et al*.[25] demonstrated a method for *PTEN* reactivation by inhibiting the MYC-WWP1 inhibitory pathway. Consequently, the identification of *PTEN* mutations and the targeting of these models both molecularly and pharmacologically hold promise as viable biomarkers for cancer patients with impaired *PTEN* function.

NF1, a tumor suppressor gene, negatively regulates the *RAS* signaling pathway. Dysfunctional *NF1* due to inactivating mutations leads to sustained increases in the intracellular levels of active RAS-GTP, resulting in prolonged activation of the RAS/RAF/MAPK signaling pathway and uncontrolled cell proliferation. Recent studies indicate that *NF1* mutations are more prevalent in NSCLC.[26] Some researchers suggest that the majority of lung cancer patients with *NF1* mutations are male smokers and that NSCLC patients with mutated *NF1* represent a distinct molecular and clinical subtype of LADC.[26,27] Studies examining *NF1* mutations across various cancers, including *NSCLC*, have primarily focused on the concurrent presence of other mutations, treatment options, and clinical evaluations.[27] While many studies have documented somatic *NF1* mutations in approximately 7–11% of LADCs, suggesting that *NF1* mutations serve as potential therapeutic targets, particularly in LADCs, information on outcomes for individuals with LSCC carrying *NF1* mutations remains scarce.[26–28] In our study, we detected *NF1* mutations in approximately 20% of patients with LSCC and found an inverse relationship with visceral pleural involvement (p = 0.09) in patients carrying *NF1* mutations—a finding not previously reported. This result warrants further investigation with a larger case series, highlighting the need to explore the clinicopathological features, coexisting mutations, treatment modalities, and clinical outcomes in LSCC patients with *NF1* mutations.

The intricate genomic landscape and carcinogenic pathways of LSCC contribute to a high mutational burden, complicating the identification of driver genes.[19] The multiple molecular alterations in LSCC hinder the generation of representative mouse models and restrict the development and implementation of targeted therapies. The high rate of driver gene mutations in LSCC often causes *FGFR1* and *PIK3CA* inhibitors to "cross-react" with wild-type genes, leading to significant toxicity reactions in clinical trials. Spoerke *et al*. discovered that LSCC models with *PTEN* mutations exhibited increased sensitivity to *PI3K* inhibitors.[29] Similarly, *PTEN* inactivation can lead to resistance to *PI3K* inhibitors.[30] Moreover, imbalances in *PI3K* and *PTEN* expression may promote immune system evasion by tumors.

The presence of concomitant mutations and their interrelationships in targeted therapy for LSCC complicate the balance between partial benefits and high toxicity.[19] These findings underscore the necessity for a comprehensive assessment of the mutation profile in LSCC patients. In our study, we identified more than one mutation in approximately 69% of the patients, with the most prevalent concomitant mutations being *TP53*, *NF1*, *PTEN*, and *FBXW7*. An examination of the relationship between these concomitant mutations and novel histopathological parameters revealed that visceral pleural involvement (p = 0.11) was less prevalent, while necrosis (p = 0.06) was more frequently observed in cases with a *TP53-NF1* mutation association. This may effectively limit local tumor spread. In patients with *TP53-PTEN* mutations, a low inflammatory response (p = 0.02) was notable, which may influence responses to immunotherapies. Although these changes may not be directly applicable to molecularly targeted therapies, they could provide insights into disease progression, treatment efficacy, survival, and prognosis. It is posited that concomitant mutations may serve as more precise biomarkers of therapeutic response compared to monogenic predictors.[1] Treatment options for LSCC are more limited than those for LADC, and patients with advanced LSCC generally have a poorer prognosis. Consequently, further studies may lead to improvements in treatment through the development of multi-target inhibitors that address the multiple driver mutations characteristic of LSCC.[19] The advent of molecular profiling in LSCC enables the molecular classification of patients in conjunction with their histopathological features, which will facilitate the formulation of personalized treatment plans, ultimately enhancing clinical prognosis and treatment outcomes.

Limitations of the study

This retrospective study was conducted in a single medical center, comprising only a small number of resected LSCC cases. The limited sample size may restrict the detection of all relevant differences; therefore, a more extensive dataset is necessary to analyze more precise molecular changes and associations in LSCC. However, we believe that the focused data from this small case set from a single center allows for a comprehensive analysis of histopathological features that may not have been available in previous studies. We also anticipate that the initial findings from our analysis could inform treatment decisions. The objective of this study was to provide a comprehensive overview of mutations and their associations with tumor histopathological information in LSCC cases exhibiting multi-gene alterations.

Conclusions

In the era of personalized medicine, treatment for LSCC lags

behind that for LADC, but new developments in this field. Careful selection of therapeutic targets is essential, as grouping multiple alterations together may lead to the oversight of true driver mutations. Similarly, drug selection and potential combinations will play a crucial role In treatment efficacy. Promising studies, including ours, will enhance understanding of the clinical relevance of suspected driver mutations. Our study identified novel mutations and their relationships, such as *NF1*, *PTEN*, and *PIK3CA,* which have not been previously reported in LSCC and may have significant therapeutic and prognostic implications. Given the molecular heterogeneity observed in LSCC, it is imperative to ascertain the mutational profile in all LSCC patients to identify driver mutations that are amenable to targeted therapy. Our data suggest that certain histopathological features may be associated with mutations, providing reliable information to enhance treatment and follow-up decisions.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Study design, histopathological review (NKÇ, İHE, İM), statistical analyses (NKÇ, SCT), statistical data interpretation (EÖ, NKÇ), original draft preparation (NKÇ, DA, SCT), review, editing (İHE, İM), and data curation (NKÇ, EÖ, SCT, DA). All authors have critically reviewed and approved the final version of the manuscript.

Ethical statement

This study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the Aydın Adnan Menderes University Faculty of Medicine Non-Invasive Clinical Research Ethics Committee (protocol no. 2024-133, approval date: 2024/25/07). Individual consent for this retrospective analysis was waived.

Data sharing statement

All data used to support the findings of this study are included in the article.

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