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A novel prognostic biomarker combining *TP53* loss-of-function and amplification of different combinations of nine genes in lung squamous cell carcinoma treated with chemotherapy

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Abstract. Lung squamous cell carcinoma (LUSC) is a prevalent subtype of lung cancer, which is primarily characterized by poor prognosis due to the lack of targeted therapies, while a large proportion of patients show limited response to chemotherapy. The present study aimed to identify predictive chemotherapy biomarkers based on genomic alterations in LUSC. Non-negative matrix factorization clustering was applied to classify patients with LUSC into distinct subgroups based on genomic alterations. Subsequently, chemotherapy efficacy was predicted via exploring gene signatures, and the results were validated using The Cancer Genome Atlas database (TCGA). A total of four distinct clusters were classified. Cluster 1 and *TP53* loss-of-function (*TP53* LOF) variations were each independently associated with poor prognosis, irrespective of clinical stage. Based on the molecular characteristics of the initial clusters, the classification was further refined by further incorporating *TP53* LOF and gene amplification (amp). Patients without *TP53* LOF, who harbored amplification of at least one of nine specified genes [*PIK3 catalytic subunit*, *cyclin D1*, *fibroblast growth factor (FGF) 19*, *FGF3*, *FGF4*, *FGF receptor 1*, *kinase insert domain receptor*, *KIT proto-oncogene* and *platelet derived growth factor receptor α*] displayed the longest progression-free survival (PFS). By contrast, patients with *TP53* LOF but lacking amplification of at least one of nine genes [Amp(9G)] exhibited the worst overall survival compared with the other subgroups. Validation in the TCGA database confirmed the prognostic significance of this classification, with Amp(9G) without *TP53* LOF predicting the most favorable PFS, and *TP53* LOF without Amp(9G) indicating the least favorable PFS. Therefore, a composite biomarker integrating Amp(9G) and *TP53* LOF was identified as a prognostic indicator for patients with LUSC treated with first-line chemotherapy. Overall, the results of the present study suggest that the absence of *TP53* LOF combined with the presence of Amp(9G) could be associated with improved response to first-line chemotherapy in LUSC.

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Abbreviations: Amp(9G), amplification of at least one of nine genes; CNV, copy number variation; CIN, chromosomal instability; FFPE, formalin-fixed paraffin-embedded; Indel, insertions and deletions; KSCC, keratinizing squamous cell carcinoma; LGR, large genomic rearrangement; LOF, loss-of-function variations; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NGS, next-generation sequencing; NKSCC, non-keratinizing squamous cell carcinoma; NMF, non-negative matrix factorization; PFS, progression-free survival; SNP, single-nucleotide polymorphisms; SNVs, single nucleotide variations; TCGA, The Cancer Genome Atlas

Key words: lung squamous cell carcinoma, cluster, gene signature, chemotherapy efficacy, prognostic marker

Introduction

Lung cancer is the leading cause of cancer-related mortality and one of the most commonly diagnosed types of cancer both in China and worldwide, accounting for an estimated 2.2 million new cases (11.4%) and 1.8 million deaths (18.0%) worldwide in 2020 (1,2). Lung squamous cell carcinoma (LUSC) is the second most prevalent histological subtype of lung cancer, accounting for 25-30% of all lung cancer cases worldwide (3). Although advances in targeted therapy and immunotherapy have improved outcomes for lung adenocarcinoma (LUAD) (4-6), the prognosis for patients with LUSC remains unsatisfactory (7).

Driver gene alterations, such as *EGFR*, anaplastic lymphoma kinase (*ALK*) and proto-oncogene tyrosine-protein kinase 1 (*ROS1*) are commonly observed in LUAD but not in LUSC (8-10). Immunotherapy is currently the preferred treatment choice for patients with advanced or metastatic LUSC, exhibiting programmed cell death ligand-1 expression of ≥1%. However, only ~20% of cases respond to this type of therapy (11-13). Consequently, chemotherapy continues to be a cornerstone of LUSC treatment (9,14). LUSC remains a

therapeutically challenging malignancy and therefore identifying reliable molecular biomarkers capable of predicting chemotherapy responsiveness and guiding clinical decision-making are of great importance.

In the present study, a large cohort of patients with LUSC were retrospectively analyzed. Non-negative matrix factorization (NMF) clustering was employed to identify the molecular subtypes of LUSC based on genomic alteration patterns. Furthermore, the predictive performance of NMF-derived genomic signatures in predicting the efficacy of chemotherapy was also evaluated. Overall, the present study aimed to identify a feasible and reliable genomic signature to identify patients with LUSC more likely to benefit from chemotherapy, thereby ensuring more precise and individualized treatment strategies.

Materials and methods

Patients and samples. Patients who met the following inclusion criteria were retrospectively analyzed: i) Patients diagnosed with LUSC according to World Health Organization criteria (15); ii) those who underwent next-generation sequencing (NGS) analysis at West China Hospital (Sichuan, China) between January 2018 and December 2020 (identification period). The observation period was measured from treatment initiation until radiographically confirmed disease progression or the administrative censoring date of January 2023; iii) aged >18 years; and iv) with tumor cell content ≥20% in formalin-fixed, paraffin-embedded (FFPE) tissue sections (3-μm thickness), as determined by an experienced pathologist on H&E-stained slides. No additional exclusion criteria were applied. Additionally, a cohort of patients with LUSC who received first-line chemotherapy were also retrospectively included to evaluate the performance of the genomic signature developed in the present study. All samples were analyzed using a 56-gene panel (LungCore; Burning Rock Biotech, Ltd). This panel was part of standard routine care and covered the entire exon regions of 56 lung cancer-related genes which was previously described in our published article (16). This was a retrospective study, which utilized existing clinical records, and all patient data and samples were rigorously de-identified prior to analysis. The full study protocol, which included a waiver of the requirement for informed consent, was reviewed and approved by the Ethics Committee of West China Hospital (Sichuan, China) and conducted according to the local ethical guidelines (approval no. 2022-1849).

DNA extraction and capture-based targeted DNA sequencing. Genomic DNA was extracted from FFPE tumor tissues using QIAsymphony® DSP DNA Mini Kit (cat. no. 937236; Qiagen GmbH), according to the manufacturer's instructions. A total of 200 ng of DNA was used for the preparation of the NGS library. DNA integrity was verified by 1.5% agarose gel electrophoresis. The concentration of DNA was quantified using the Qubit dsDNA HS Assay Kit on a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Inc.). DNA was fragmented using the Covaris M220 system (Covaris, LLC). The fragmented DNA was end-repaired and 3'-adenylated in a combined reaction, followed by adapter ligation using T4 DNA ligase (Burning Rock Biotech, Ltd.). Both enzymatic reactions were carried out in PCR tubes with thermal cycling under a heated lid (85˚C). DNA fragments ranging from 200 to 400 bp were purified using the Agencourt AMPure XP Kit (Beckman Coulter, Inc.), followed by hybridization with capture probes; specifically, 50 ng/μl of capture probe baits were co-incubated with DNA fragments in a PCR instrument at 65˚C for 16-24 h. This was followed by hybrid selection with magnetic beads and PCR amplification with DNA polymerase (Burning Rock Biotech, Ltd.). PCR amplification was performed with an annealing temperature of 60˚C for 12 cycles with Illumina-provided i5/i7 primers. Library quality was assessed using the Qubit dsDNA HS Assay Kit (cat. no. Q32854; Invitrogen; Thermo Fisher Scientific, Inc.) and the Agilent 2100 Bioanalyzer System (Agilent Technologies, Inc.). Indexed libraries were primarily sequenced on the Miseq (Illumina, Inc.) platform. A subset of 66 libraries was sequenced on the MiniSeq (cat. no. SY-410-1003; Illumina, Inc.) platform due to instrument availability. The loading concentration of the final library was 14 pM for Miseq and 1.5 pM for MiniSeq. All sequencing was performed using 150-bp paired-end reads.

Sequencing data analysis. Sequencing data were mapped to the human reference genome (hg19) using Burrows-Wheeler aligner 0.7.10 (https://sourceforge.net/projects/bio-bwa/files/) (17). Local realignment and variant calling were performed using the Genome Analysis Toolkit version 3.2 (18) and VarScan software 2.4.3 (Genome Institute at Washington University) (19). Variants were filtered using the VarScan filter pipeline, requiring a minimum depth of 100x. From FFPE tumor tissue, at least five supporting reads for short insertions and deletions (indels) and eight for single nucleotide variations (SNVs) were required. Variants with a population frequency of >0.1% were considered as common single-nucleotide polymorphisms and excluded according to the Exome Aggregation Consortium (https://gnomad.broadinstitute.org), 1000 Genomes Project (https://www.internationalgenome.org/), dbSNP (https://www.ncbi.nlm.nih.gov/snp/) and ESP6500SI-V2 (https://genome.ucsc.edu/cgi-bin/hgTables?db=hg19&hgta_group=varRep&hgta_track=evsEsp6500&hgta_table=evsEsp6500&hgta_doSchema=describe+table+schema) databases. The remaining variants were annotated using ANNOVAR (2016-02-01 release) (20) and SnpEff v3.6 (21). DNA translocation was detected using Factera v.1.4.3 (22). Indels, copy number variations (CNVs) and large genomic rearrangements (LGRs) were identified as previously described (23,24).

NMF analysis. Data clustering was performed using NMF based on Euclidean distance (25,26). Non-synonymous SNVs, indels, CNVs and structural variations were included in the NMF clustering analysis. Patients harboring only synonymous SNVs or undetectable genomic alterations were excluded from the analysis. The R package 'NMF' (version 0.22.0) was implemented in R (version 3.4.0; R Foundation for Statistical Computing) to estimate the optimal factorization rank using Lee and Seung's algorithm with 2:8 ranks (27). A marked decrease in the cophenetic correlation coefficient was observed between ranks 4 and 5 (28), therefore rank 4 was selected as the optimal value, thus resulting in four subgroups.

Validation data collection. Sequencing data from patients with LUSC were downloaded from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/) database using the R package 'TCGAbiolinks'. The clinical characteristics of patients, including age, sex tumor stage, progression-free survival (PFS) and patient outcomes were also collected. The prognostic value of the NMF-based model was validated in the TCGA LUSC cohort.

Statistical analysis. All analyses were performed using R statistics packages (R v3.4.0; Posit Software). The differences between the two groups were compared using Fisher's exact test for categorical variables. For comparisons involving more than two groups, the Kruskal-Wallis test was first applied. When the overall test was significant, pairwise post-hoc comparisons were performed using the Wilcoxon rank-sum test with Holm correction to control for multiple testing. PFS was assessed using the Kaplan-Meier method, and differences between survival curves were compared using the log-rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics. A total of 317 patients with LUSC were included in the present study. The majority of patients were men (89.9%; 285/317), aged ≥60 years (67.2%; 213/317) and were former or current smokers (57.4%; 182/317). Histologically, 55.2% (175/317) of tumors were classified as keratinizing squamous cell carcinoma (KSCC), 28.7% (91/317) as non-KSCC (NKSCC), while 16.1% (51/317) were of unknown subtype. The distribution of tumor stage varied, with 3.2% (10/317) of patients diagnosed with stage I, 6.6% (21/317) with stage II, 28.7% (91/317) with stage III, 27.8% (88/317) with stage IV, while 33.8% (107/317) of cases were of unknown stage. Among the 317 patients, 35 received first-line chemotherapy. Of these, 91.4% (32/35) were men and 88.6% (31/35) were former or current smokers. Histologically, 65.7% (23/35) were diagnosed with KSCC, 25.7% (9/35) with NKSCC and 8.6% (3/35) were of unknown subtype. In terms of disease stage, 5.7% (2/35) had stage I disease, 25.7% (9/35) stage II, 34.3% (12/35) stage III, 28.6% (10/35) stage IV and 5.7% (2/35) had unknown stage. The detailed clinical characteristics of patients are presented in Table I. The two cohorts showed no statistically significant differences in terms of age (P=0.101), sex (P=1.000), subtype (P=0.557), T stage (P=0.948), N stage (P=0.180) and M stage (P=0.341). However, marginally significant differences were observed in terms of smoking history (P=0.048) and tumor stage (P=0.049). In addition, the clinical characteristics of patients from TCGA database and those who received radiochemotherapy are shown in Tables SI and SII.

Genomic alterations of LUSC. Genomic alterations, including non-synonymous SNVs, indels, CNVs, gene fusions and LGRs, were detected in 98% (311/317) of patients (Fig. 1A). *TP53* was the most commonly altered gene (91%; 287/317) followed by *PIK3 catalytic subunit* (*PIK3CA*; 46%; 145/317), *cyclin dependent kinase inhibitor 2A* (*CDKN2A*; 24%; 77/317) and *EGFR* (17%; 53/317). CNVs were identified in 209 cases, with the most prevalent CNVs detected in *PIK3CA* (38%; 120/317), *fibroblast growth factor (FGF) receptor 1* (*FGFR1*; 15%; 48/317) and genes located at chromosome 11q13 (11%; 35/317), including *cyclin D1* (*CCND1*; 11%; 35/317), *FGF3* (9%; 28/317), *FGF4* (8%; 25/317) and *FGF19* (10%; 33/317). SNVs and indels were detected in 305 patients, and the highest frequency was recorded in *TP53* (90%; 285/317), followed by *CDKN2A* (22%; 71/317), *PIK3CA* (14%; 45/317), *PTEN* (14%; 43/317), *EGFR* (11%; 36/317) and *Erb-B2 receptor tyrosine kinase 4* (*ERBB4*; 10%; 32/317). Additionally, nine fusion events were identified, including *EML4-ALK* fusions in two patients, and *SEC61G-EGFR*, *CASC21-MYC*, *FGFR1-ADAM9*, *FGFR3-TACC3*, *C12orf66-KIT* and *FGFR3-TACC3*, *FGFR1-IGFBPL1* fusions in one patient each. Two patients carried LGRs, including *RB transcriptional corepressor 1* (*RB1*) in one case and *TP53* in the other.

NMF-based clustering analysis. NMF-based clustering was performed to classify patients according to their genomic profile. Excluded from the analysis were two patients harboring synonymous SNVs and six with undetectable genomic alterations. Therefore, NMF analysis was conducted for a total of 309 patients with LUSC. These patients were classified into the following four distinct clusters (Fig. 1A): i) Cluster 1 (C1) consisted of 129 patients characterized by *TP53* alterations; ii) Cluster 2 (C2) included 116 patients with *PIK3CA* amp; iii) Cluster 3 (C3) composed of 33 patients characterized by amp of *CCND1, FGF19, FGF3 and FGF4*; and iv) Cluster 4 (C4) included 31 patients characterized by amp of *FGFR1*, *kinase insert domain receptor* (*KDR*), *KIT proto-oncogene* (*KIT*) and *platelet derived growth factor receptor α* (*PDGFRA*). Among the four clusters, C3 and C1 exhibited the highest and lowest CNV frequency, respectively (Fig. 1B).

Association between different clusters and survival outcomes in patients with LUSC. The association between NMF-based molecular clusters and survival outcomes was subsequently investigated. Among the 317 patients with LUSC, 35 patients who received first-line chemotherapy and had available PFS data were included in a retrospective cohort. A single patient was excluded due to the undetectable genetic alterations, leaving 34 patients for NMF analysis. Of the aforementioned patients, 13, 10, 5 and 6 were allocated into the C1, C2, C3 and C4 clusters, respectively (Fig. 2A). No statistically significant difference in PFS was observed among the four clusters (P=0.12; Fig. 2B). Pairwise comparisons further confirmed that PFS did not differ significantly among C2, C3 and C4 (all P>0.5; Fig. 2B). Given their comparable chemotherapy outcomes and the shared feature of gene amplification, C2, C3 and C4 were therefore merged in a data-driven manner for subsequent analyses. Notably, patients in C1 exhibited significantly shorter PFS compared with those in C2/3/4 [3.5 vs. 12.5 months; P=0.018; hazard ratio (HR) =0.35; 95% confidence interval (CI): 0.14-0.87; Fig. 2C). Additionally, tumor stage distribution was similar among the clusters, thus indicating a comparable background in tumor stage. (P=0.415; Fig. S1A; and P=0.293; Fig. S1B).

TP53 loss-of-function (LOF) variations combined with NMF-based molecular clustering for PFS prediction. The LOF alterations in *TP53* are known contributors to chemotherapy resistance in several types of cancer (29).

Table I. Baseline characteristics of 317 patients with lung squamous cell carcinoma and chemotherapy regimens in the chemotherapy cohort.

Characteristic	Total (n=317)	Chemotherapy (n=35)	P-value
Age, years			0.101
Mean, SD	64.1 (9.7)	61.2 (9.6)	
Median, IQR	64.0 (56.0, 71.0)	63.0 (53.5, 67.0)	
Sex			1.000
Female	32 (10.1)	3 (8.6)	
Male	285 (89.9)	32 (91.4)	
Subtype			0.557
KSCC	175 (55.2)	23 (65.7)	
NKSCC	91 (28.7)	9 (25.7)	
Unknown	51 (16.1)	3 (8.6)	
Smoker			0.048
No	57 (18.0)	3 (8.6)	
Yes	182 (57.4)	31 (88.6)	
Unknown	78 (24.6)	1 (2.9)	
Stage			0.049
I	10 (3.2)	2 (5.7)	
II	21 (6.6)	9 (25.7)	
III	91 (28.7)	12 (34.3)	
IV	88 (27.8)	10 (28.6)	
Unknown	107 (33.8)	2 (5.7)	
T stage			0.948
T1	12 (3.8)	2 (5.7)	
T2	50 (15.8)	9 (25.7)	
T3	47 (14.8)	9 (25.7)	
T4	93 (29.3)	14 (40)	
Unknown	115 (36.3)	1 (2.9)	
N stage			0.180
N0	39 (12.3)	12 (34.3)	
N1	26 (8.2)	5 (14.3)	
N2	74 (23.3)	8 (22.9)	
N3	59 (18.6)	9 (25.7)	
Unknown	119 (37.5)	1 (2.9)	
M stage			0.341
M0	121 (38.2)	23 (65.7)	
M1	79 (24.9)	10 (28.6)	
Unknown	117 (36.9)	2 (5.7)	
Chemotherapy regimens	-		-
Taxane plus platinum		23 (65.7)	
Taxane (Paclitaxel)		6 (17.1)	
Gemcitabine		1 (2.9)	
Gemcitabine plus Platinum		4 (11.4)	
Gemcitabine plus cisplatin-Paclitaxel plus cisplatin		1 (2.9)	

Values are expressed as n (%) unless otherwise specified. LUSC, lung squamous cell carcinoma; KSCC, keratinizing squamous cell carcinoma; NKSCC, non-keratinizing squamous cell carcinoma; T, tumor; N, node; M, metastasis.

In the present study, the prognostic effect of *TP53* LOF in 35 patients with LUSC treated with first-line chemotherapy was explored. In the present chemotherapy cohort, all 29 patients (83%) harboring *TP53* mutations were annotated

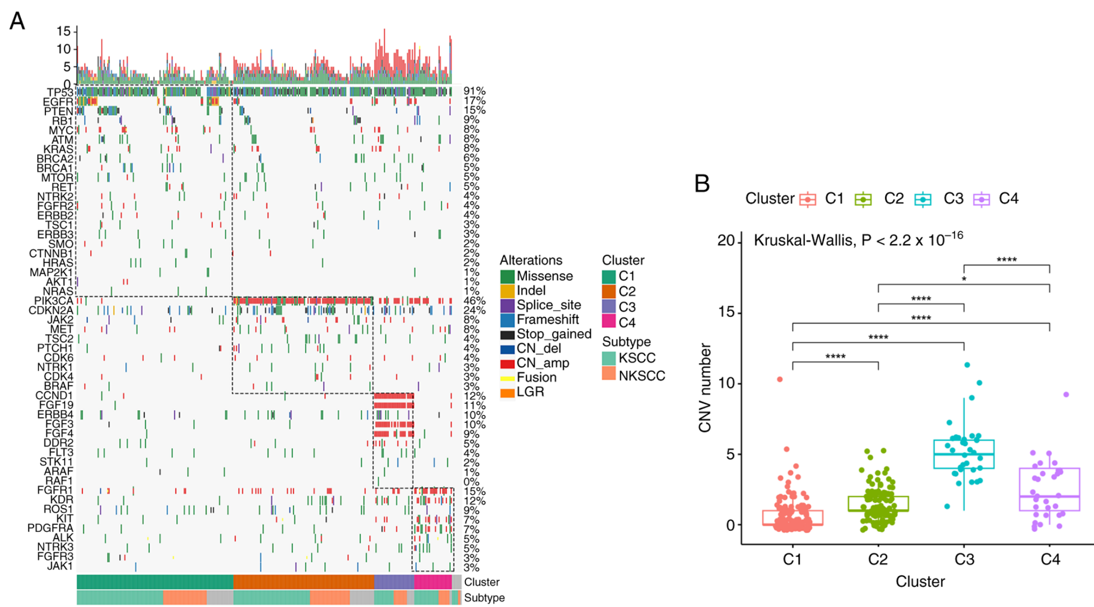

Figure 1. NMF-based molecular clustering of 317 patients with LUSC. (A) NMF-based molecular clustering of patients with LUSC, classified into four clusters (C1, C2, C3 and C4). (B) Differences in the number of copy number variants among the four molecular clusters (C1-4) were first assessed using the Kruskal-Wallis test, followed by pairwise comparisons using the Wilcoxon rank-sum test with Holm correction. *P<0.05, ****P<0.0001. NMF, non-negative matrix factorization; LUSC, lung squamous cell carcinoma; C, cluster; Indel, insertion and deletion; CN, copy number; CNV, copy number variation; LGR, large genomic rearrangement; KSCC, keratinizing squamous cell carcinoma; NKSCC, non-keratinizing squamous cell carcinoma; del, deletions; amp, amplification.

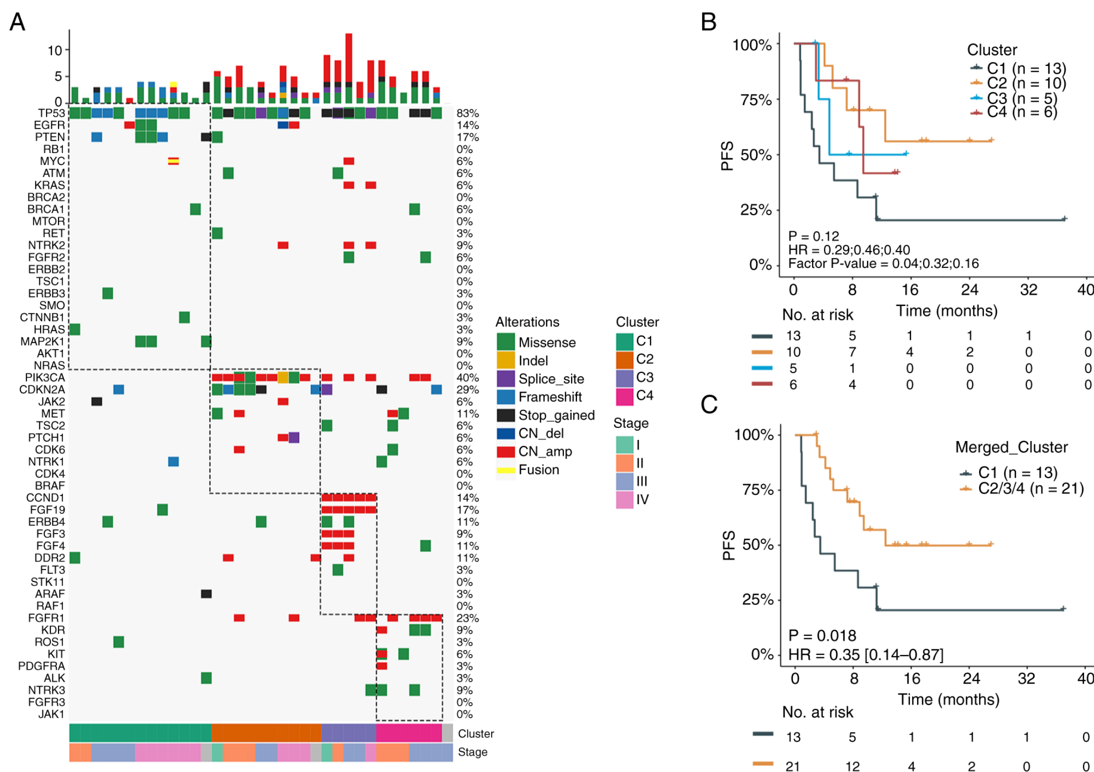

Figure 2. Associations between NMF-based molecular clustering and PFS in patients with lung squamous cell carcinoma treated with first-line chemotherapy. (A) NMF-based molecular clustering of patients who received first-line chemotherapy, classified into C1, C2, C3 and C4. (B) PFS between C1-C4 is presented. (C) PFS between C1 and C2/3/4 is presented. NMF, non-negative matrix factorization; C, cluster; PFS, progression-free survival; HR, hazard ratio.

as LOF according to the OncoKB database (https://www.oncokb.org/gene/TP53#tab=FDA). However, patients with *TP53* mutations showed no significant difference in PFS compared with *TP53*-wild-type patients (P=0.835; Fig. S2). Considering that truncating alterations (frameshift, splice-site, nonsense mutations or copy number deletions) are expected to result in complete functional loss through nonsense-mediated mRNA decay or production of severely

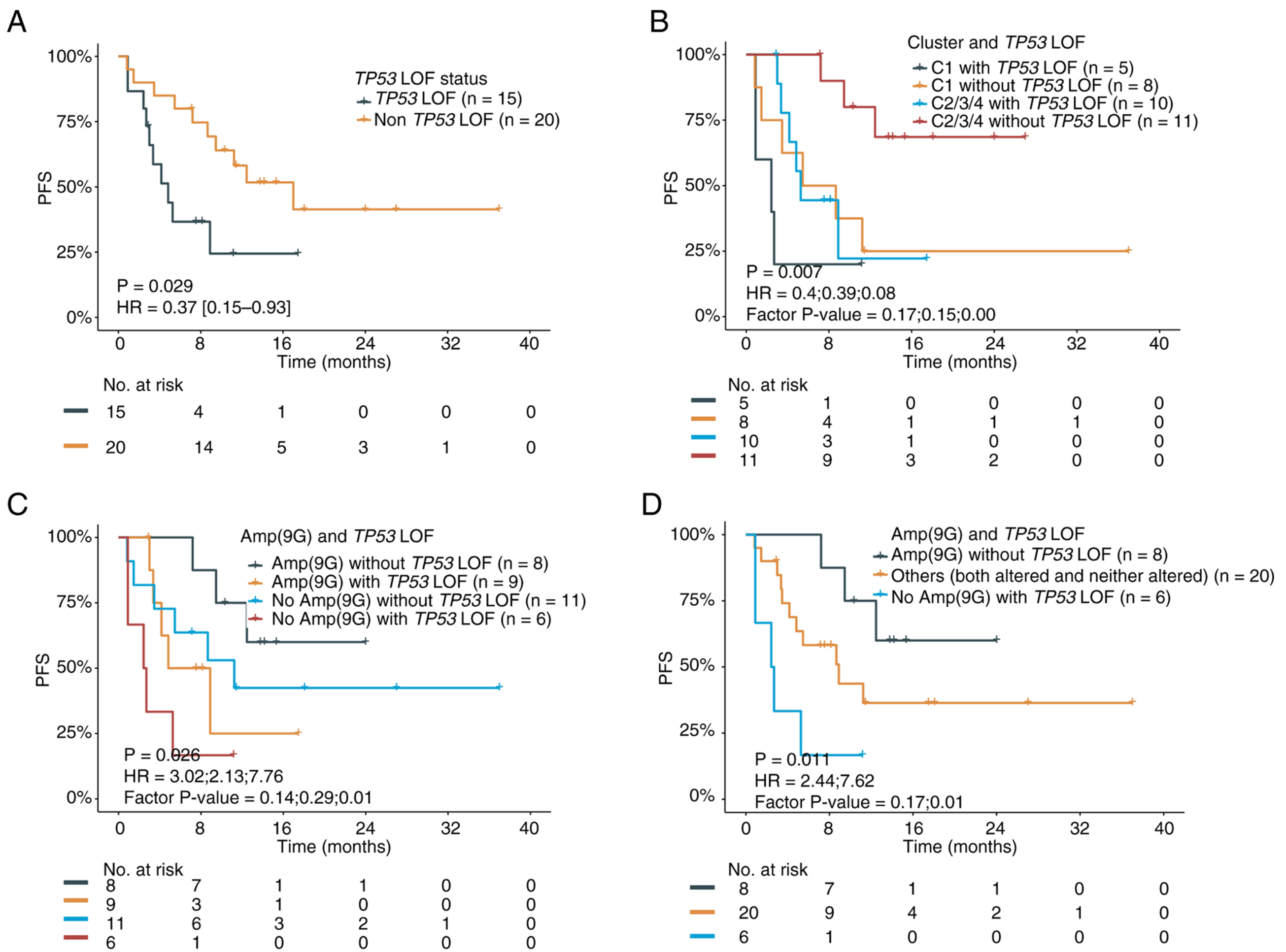

Figure 3. Prognostic value of *TP53* LOF alterations alone or in combination with non-negative matrix factorization-based molecular clustering or gene amp status in patients with lung squamous cell carcinoma who received first-line chemotherapy. (A) PFS stratified by *TP53* LOF status. (B) PFS comparison among four subgroups: C1 with *TP53* LOF, C1 without *TP53* LOF, C2/3/4 with *TP53* LOF and C2/3/4 without *TP53* LOF. (C) PFS comparison among patients harboring Amp(9G) with *TP53* LOF, Amp(9G) without *TP53* LOF, *TP53* LOF without Amp(9G) and those without *TP53* LOF and Amp(9G). (D) PFS comparison among patients harboring *TP53* LOF without Amp(9G), Amp(9G) without *TP53* LOF and others [including those with Amp(9G) and *TP53* LOF and those without Amp(9G) and *TP53* LOF]. LOF, loss-of-function; PFS, progression-free survival; C, cluster; amp, amplification; Amp(9G), amplification of at least one of nine genes.

truncated proteins, whereas missense mutations may exert heterogeneous effects including partial LOF, dominant-negative or gain-of-function (30,31), *TP53* LOF was defined in the present study exclusively as truncating alterations and did not include missense mutations in the LOF category. Using this definition, the results revealed that patients with *TP53* LOF (n=15) exhibited significantly shorter median PFS (mPFS) compared with those without *TP53* LOF (4.8 vs. 17 months; P=0.029; HR=0.37; 95% CI: 0.15-0.93; Fig. 3A). No significant difference was observed in the distributions of *TP53* LOF across all clusters (P=0.389; Fig. S3A), nor specifically among C2, C3 and C4 clusters (P=0.288) and the distribution of *TP53* LOF was also similar between C1 and C2/3/4 clusters (P=0.728; Fig. S3B). A comparison of *TP53* background can be performed across these clusters. Furthermore, the combined predictive value of *TP53* LOF and NMF-based molecular clustering in patients with LUSC was evaluated. The results demonstrated that patients in C1 with *TP53* LOF exhibited worse prognosis (mPFS=2.4 months) compared with C1 without *TP53* LOF (mPFS=7.1 months), C2/3/4 with *TP53* LOF (mPFS=5.3 months) and C2/3/4 without *TP53* LOF (mPFS not reached; Fig. 3B). Additionally, to refine the clustering approach, the status of *TP53* alterations and gene amps were considered, thus resulting in the classification of 34 patients into four subtypes. Notably, patients without *TP53* LOF, but with amp of at least one of the nine specified genes (*PIK3CA*, *CCND1*, *FGF19*, *FGF3*, *FGF4*, *FGFR1*, *KDR*, *KIT* and *PDGFRA*) displayed the longest mPFS (not reached) compared with those with *TP53* LOF but without Amp(9G), who exhibited the shortest PFS (mPFS=2.6 months; Fig. 3C). Consistent results were observed when patients with Amp(9G) and *TP53* LOF were merged with those without Amp(9G) and *TP53* LOF into a single subgroup (Fig. 3D). Multivariable Cox analysis demonstrated that the Amp(9G) combined with *TP53* LOF-based grouping was independently associated with PFS after adjustment for tumor stage (Fig. S4).

Validation of NMF-based signature. To validate the aforementioned findings, DNA sequencing data and clinicopathological characteristics of patients with LUSC were downloaded from TCGA database. The analysis included only patients who received chemotherapy, resulting in a

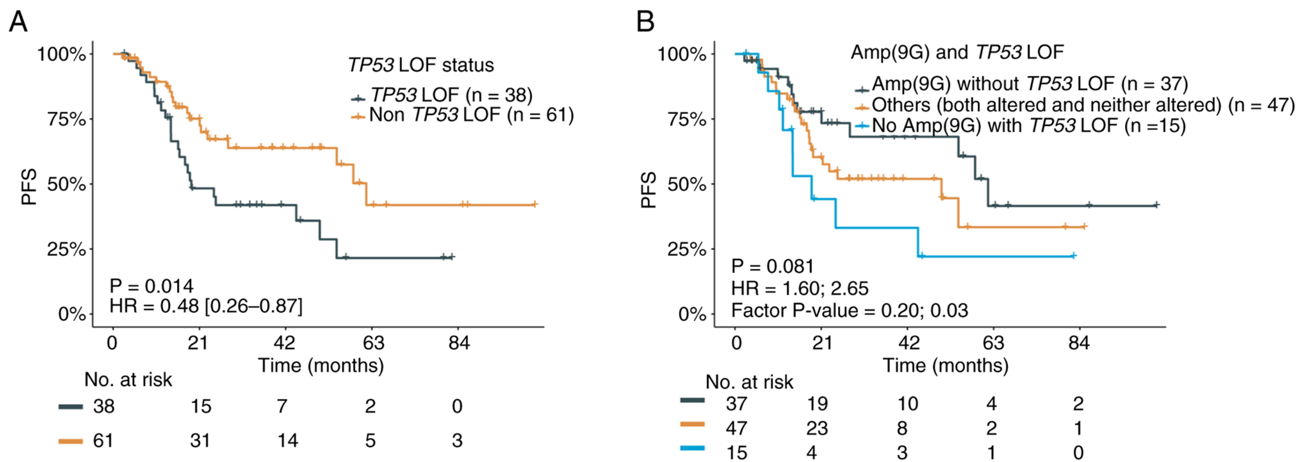

Figure 4. Validation of the prognostic potential of *TP53* LOF alone or combined with Amp(9G) in the TCGA LUSC cohort treated with chemotherapy. (A) PFS stratified by *TP53* LOF status in the TCGA LUSC cohort. (B) PFS comparison among patients harboring *TP53* LOF without Amp(9G), Amp(9G) without *TP53* LOF and others [including those with Amp(9G) and *TP53* LOF, and those without Amp(9G) and *TP53* LOF]. LOF, loss-of-function; TCGA, The Cancer Genome Atlas; LUSC, lung squamous cell carcinoma; PFS, progression-free survival; amp, amplification; Amp(9G), amplification of at least one of nine genes.

validation cohort of 99 patients (TCGA-LUSC cohort). The results showed that *TP53* LOF was associated with shorter PFS compared without *TP53* LOF (19.0 vs. 61.6 months; P=0.014; HR=0.48; 95% CI: 0.26-0.87; Fig. 4A). NMF analysis further stratified the TCGA-LUSC cohort into the same four clusters (Fig. S5). Furthermore, evaluation of the combined *TP53* LOF and Amp(9G) revealed that patients with Amp(9G) but without *TP53* LOF had favorable mPFS (61.6 months), while those with *TP53* LOF and no Amp(9G) had worse mPFS (18.7 months; Fig. 4B). The Amp(9G) and *TP53* LOF signature remained an independent prognostic factor for PFS in multivariable analysis after controlling for tumor stage (Fig. S6).

To further investigate the feasibility of this signature in predicting the efficacy of first-line chemotherapy in LUSC, a total of 24 patients with LUSC treated with first-line radiochemotherapy were respectively analyzed. These patients were allocated into three groups based on the Amp(9G) and *TP53* LOF status. Therefore, *TP53* LOF combined with Amp(9G) was not associated with PFS (P=0.419; Fig. S7), indicating that its prognostic value could be specific to patients receiving first-line chemotherapy alone, and not to those treated with concurrent first-line radiochemotherapy.

Discussion

Although targeted therapy and immunotherapy have markedly improved cancer treatment (32,33), their efficacy in patients with LUSC remains limited (34,35). Consequently, chemotherapy still plays a notable role in the management of LUSC (36). Therefore, the identification of reliable biomarkers for predicting chemotherapy response and clinical response is urgently needed.

Although comparative analysis revealed marginally significant differences in terms of smoking history and tumor stage between the overall cohort and the chemotherapy cohort, no significant differences were observed in individual TNM components (Table I). These results indicated a comparable background in TNM stage between the two cohorts. Moreover, clinical stage was included in the multivariate analyses, in which the identified biomarker (*TP53* LOF and Amp(9G) remained an independent predictor of prognosis, indicating that the observed prognostic value is unlikely to be driven by these baseline differences. In the present study, NMF analysis was performed to genomic profiling data to cluster molecular subtypes. NMF is a commonly used clustering approach for identifying characteristic gene modules in cancer and has been successfully applied to stratify prognosis in several types of cancer, including pancreatic cancer, head and neck squamous carcinoma, hepatocellular carcinoma and glioblastoma (37-40). Although several molecular subtypes associated with prognosis in LUSC have been previously reported (41,42), to the best of our knowledge, the present study was the first to employ NMF-based analysis of genomic alterations to assess chemotherapy efficacy and predict prognosis in patients with LUSC.

Studies have reported that *TP53*, *CDKN2A*, *PTEN*, *PIK3CA*, *Kelch like ECH associated protein 1*, *mixed-lineage leukemia 2*, *major histocompatibility complex class I A*, *nuclear factor erythroid 2-related factor 2*, *NOTCH1* and *RB1* are notably altered in LUSC, with *TP53* alterations present in the majority of cases (8,43-46). Consistent with the previous reports, in the present study, *TP53* alterations were detected in 91% of patients with LUSC. In addition, *PIK3CA* (46%), *CDKN2A* (24%) and *PTEN* (15%) were among the most commonly altered genes. In LUSC, 8p11 (*FGFR1* and *Wolf-Hirschhorn syndrome candidate 1-like 1*), 7p11 (*EGFR*), 11q13 (*CCDN1*) and 4q12 (*KDR*, *KIT* and *PDGFRA*) amps have also been frequently reported (8,45,47). In the present study, *FGFR1* amp was identified in 16% of patients with LUSC, which was consistent with previous studies (48,49). Overall, the aforementioned findings indicated that there was no patient selection bias in the present study.

A total of 309 patients with LUSC were classified into four molecular clusters based on their genomic alterations using NMF analysis, including C1 (*TP53* alterations), C2 (*PIK3CA* amp), C3 (*CCND1*, *FGF19*, *FGF3* and *FGF4* amp) and C4 (*FGFR1*, *KDR*, *KIT* and *PDGFRA* amp). Subsequently, the

present study investigated whether these NMF-based molecular clusters could predict the efficacy of chemotherapy in patients with LUSC. LUSC is characterized by a high rate of CNVs compared with other types of cancer (50). Recurrent amplifications involving *SOX2*, *PIK3CA*, *PDGFRA/KIT*, *FGFR1*, *CCND1 and FGF3/4/19* are commonly observed in LUSC (50,51). CNVs represent a hallmark of chromosomal instability (CIN) in cancer (52). More particularly, Teixeira *et al* (53) indicated that CIN, characterized by widespread copy number alteration, could be detected at the pre-malignant stage. CIN has been shown to exert dual effects on tumor progression. Therefore, although moderate levels of CIN can promote tumor evolution via increasing intratumoral heterogeneity and contributing to therapeutic resistance (54), excessive CIN can exceed cellular tolerance to genomic stress and induce tumor cell death, particularly in the context of DNA damage-based therapies such as chemotherapy (55).

TP53, a well-established tumor suppressor gene, is commonly mutated across a wide spectrum of cancer types. As a sequence-specific transcription factor, TP53 protein plays a key role in regulating the expression of adjacent genes via binding to specific DNA sequences (56,57). *TP53* deficiency can lead to multifaceted oncogenic consequences, including impaired cell cycle control, compromised apoptotic signaling and enhanced genomic instability (57,58). Different types of cancer can actively evade chemotherapy-induced DNA damage-dependent cell senescence and apoptosis through synergistic interactions. Emerging evidence has suggested that *TP53* LOF drives tumor metastasis, disease progression and resistance to chemotherapy (29,59-61). Consistent with these findings, in the present retrospective cohort, *TP53* LOF, arising from frameshift mutations, splice site mutations, copy number deletions and nonsense mutations, was significantly associated with shorter mPFS in patients with LUSC treated with first-line chemotherapy. This association was also independently validated in the TCGA-LUSC cohort, indicating that *TP53* LOF could be a robust predictor of worse prognosis in patients with LUSC receiving first-line chemotherapy.

In the present study, both *TP53* LOF and NMF-based molecular clustering could predict chemotherapy efficacy in LUSC. In addition, whether *TP53* combined with NMF-based molecular clustering could improve prognostic stratification was subsequently explored. For clinical practicality, clusters C2/3/4, each characterized by gene amp and similar PFS behavior, were grouped together. Therefore, a simplified biomarker signature based on Amp(9G) (*PIK3CA*, *CCND1*, *FGF19*, *FGF3*, *FGF4*, *FGFR1*, *KDR*, *KIT* and *PDGFRA*) combined with *TP53* status was established. Using the aforementioned approach, the results demonstrated that Amp(9G) without *TP53* LOF exhibited the most favorable PFS, while patients with *TP53* LOF without Amp(9G) experienced the worst PFS, both in the retrospective and TCGA validation cohorts. This finding could be because increased CIN mediated by gene amp could represent a specific vulnerability of tumor cells. Supra-threshold CIN can potentially induce tumor cell death (62,63). Notably, the combination of Amp(9G) and *TP53* LOF failed to predict prognosis in radiochemotherapy-treated patients with LUSC. These findings suggested that Amp(9G) combined with *TP53* LOF could serve as a feasible tool for predicting prognosis in patients with LUSC receiving chemotherapy in clinical practice.

However, the present study has some limitations that should be acknowledged. Although significant differences in PFS were observed among groups stratified by Amp(9G) combined with *TP53* LOF in the retrospective LUSC cohort, statistical significance was not reached in the TCGA-LUSC cohort, despite a consistent trend. The aforementioned discrepancy could reflect differences in the populations studied, with the retrospective cohort comprising Chinese patients and the TCGA-LUSC cohort representing a Western population. Additionally, the distribution of tumor stage was different between the two cohorts with stage III/IV predominating in the retrospective cohort and stage I/II being more common in the TCGA-LUSC cohort. The chemotherapy cohort was relatively small and included heterogeneous platinum-based regimens, which precluded detailed stratified or multivariable analyses according to specific treatments and may introduce residual confounding. Therefore, larger, prospective cohort studies are needed to further validate the predictive and prognostic value of the Amp(9G) and *TP53* LOF signature in patients with LUSC treated with first-line chemotherapy.

In conclusion, in the present study, NMF-based molecular clustering to genomic data was applied to predict the efficacy of first-line chemotherapy in patients with LUSC. An applicable biomarker signature encompassing Amp(9G) and *TP53* LOF was developed and the performance of this signature was validated in the TCGA-LUSC cohort and patients who received radiochemotherapy. The results suggested that Amp(9G) without *TP53* LOF could serve as a favorable prognostic biomarker for patients with LUSC receiving chemotherapy.

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Availability of data and materials

The raw sequencing data generated in the present study can be found in the officially designated repository in China, Genome Sequence Archive for Human (GSA-Human: HRA016539) that are publicly accessible at: https://ngdc.cncb.ac.cn/gsa-human/browse/HRA016539. The datasets are under controlled access.Researchers interested in obtaining the data can apply for permission through the GSA-Human application system. Upon approval by the Data Access Committee (DAC), authorized users can download the data. The verification data generated and analyzed in the present study are available from the PanCanAtlas dataset, which is publicly available at https://gdc.cancer.gov/about-data/publications/pancanatlas.

Authors' contributions

YL participated in the study design and data curation and was responsible for writing original draft. TH and HL contributed to the data analysis and methodological validation. HD wrote the original manuscript with YL and performed bioinformatics data processing. LQ and YZ contributed to the data acquisition and visualization. GZ designed the methodology and provided supervision. YT contributed to the conceptualization, revised the manuscript and performed the project administration. YL and TH confirm the authenticity of the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

This retrospective study utilized existing clinical records, and all patient data and samples were rigorously de-identified prior to analysis. The tissue analysis using a 56-gene panel in the retrospective study was part of standard clinical care, not a research-specific assay. Therefore, the full study protocol which included a waiver of the requirement for informed consent based on the use of anonymized data was reviewed and approved by the Ethics Committee of West China Hospital (approval no. 2022-1849).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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