Identification of tumor antigens and immune subtypes in lung squamous cell carcinoma for mRNA vaccine development

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Background: Cancer vaccines are therapies that activate the patient's own immune system by inoculating the patient with cancer-specific antigens to identify and clear cancer cells. Messenger ribonucleic acid (mRNA) vaccines have received much attention because of their ease of synthesis, relative affordability, and long-term safety, but have not been studied in lung squamous cell carcinoma (LUSC). Thus, the identification of tumor antigens is necessary to facilitate the development of mRNA vaccines for LUSC.

Methods: Genes with significant copy number amplification, the presence of gene mutations in LUSC, and genes associated with survival were identified as potential tumor antigens. Subsequently, the genes significantly correlated with the level of infiltration of 3 antigen-presenting cells in LUSC were identified as LUSC tumor antigens. Unsupervised clustering and immune profiling analysis were used to identify potential suitable patients for mRNA vaccines. Lastly, drug sensitivity analysis was used to explore individualized treatment options for mRNA vaccines suitable and unsuitable patients.

Results: In this study, among the highly mutated genes in LUSC, we found that bone morphogenetic protein 5 (BMP5) and claudin 5 (CLDN5) expression were positively correlated with antigen-presenting cell infiltration, which indicates that these 2 genes have potential for development as mRNA cancer vaccines. Further, the immune landscape analysis identified different immune subtypes. Our findings provide a reference for the development of treatment strategies and the prediction of treatment responsiveness.

Conclusions: Overall, our study provides a new perspective on the development of mRNA vaccines for LUSC and highlights the importance of individualized therapy.

Keywords: Lung squamous cell carcinoma (LUSC); messenger ribonucleic acid vaccine (mRNA vaccine); tumor antigens

Introduction

Lung cancer is the leading cause of cancer-related death (1,2). The emergence of targeted therapy and immunotherapy in recent years has greatly improved the overall survival (OS) of patients; however, drug resistance and the low expression of immune checkpoints are still key issues that need to be solved (3). The mortality rate of lung squamous cell carcinoma (LUSC) patients continues

to increase due to a lack of effective targeted drugs (3,4). Drug resistance in lung cancer is mainly caused by the heterogeneity of cancer cells and metabolic reprogramming-driven microenvironment adaptation phenotypes (5). Thus, it is very important to focus on the development of individualized treatment strategies and to find new treatments for lung cancer to address existing problems.

Cancer vaccines and chimeric antigen receptor T

(CAR-T) cell therapy are emerging hot topics in cancer treatment. Cancer vaccines mainly activate the immune system of cancer patients to recognize and remove cancer cells by vaccinating specific antigens of cancer, while CAR-T therapy mainly enhances the anti-tumor immunity of patients by engineering the T cells extracted from the patients themselves and expanding them before transfusion (6-9). Cancer vaccines have received much attention in recent years due to their economical nature, and clinical studies have been conducted in the treatment of a variety of cancers (10-14). Cancer vaccines are mainly divided into messenger ribonucleic acid (mRNA) vaccines, peptide vaccines, and lentiviral vaccines, and their main difference is the different vectors that deliver the cancer antigens (6,15).

The mRNA tumor vaccine is generally prepared by using the template mRNA of translated proteins and injected into the body to synthesize specific antigenic proteins as "targets" through the protein synthesis system of human cells to induce an immune response to the "targets" and then target the tumor cells. mRNA vaccines have attracted much attention in the study of a variety of cancer vaccines because of their easy synthesis and economic advantages (16). mRNA vaccines do not integrate into the genome and can also be degraded by mRNA enzymes *in vivo* with good long-term safety (9,17), and their safety has also been confirmed in the process of counteracting coronavirus disease 2019 (18). Meanwhile, mRNA has the limitations of causing strong immune response and high storage conditions. However, to date no relevant studies on mRNA cancer vaccines in the treatment of LUSC have been conducted.

In this article, we used bioinformatics methods based on the specificity and immunogenicity of the vaccine to identify potential LUSC tumor antigens and revealed the immune landscape of patients with LUSC through a subsequent analysis, identified the characteristics of the population suitable for mRNA vaccination, provided a new perspective for the development of mRNA vaccines for LUSC, and emphasized the importance of individualized therapy. We present the following article in accordance with the STREGA reporting checklist (available at https://jtd. amegroups.com/article/view/10.21037/jtd-22-1113/rc).

Methods

Acquisition of public data on LUSC

The LUSC transcriptome and mutation data used in this study were downloaded from University of California, Santa Cruz website (https://xena.ucsc.edu/) [cohort: GDC The Cancer Genome Atlas (TCGA)-LUSC]. High-throughput sequence (HTSeq)-Fragments per kilobase of exon model per million mapped fragments (FPKM), somatic mutation data, and sample clinical information were downloaded (19). Masked copy number segment data for LUSC were downloaded from TCGA (https://portal.gdc.cancer.gov/). Genes associated with OS in LUSC were obtained from the Gene Expression Profiling Interactive Analysis 2 (GEPIA 2; http://gepia2.cancer-pku.cn/#survival) database. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Identification of potential tumor antigens

A copy number variation analysis was performed using genomic identification of significant targets in cancer (GISTIC) 2.0 software (20). The human genome reference consortium human build 38 (GRCh38) was used as the reference genome, and the significance threshold was set at 0.01. Software default values were used for the remaining parameters. The genomic localization of all significantly amplified genes was visualized using the "RCircos" R package. The functional enrichment analysis of genes with significantly amplified copy numbers was performed using the "clusterprofiler" R package (21). All the mutated genes were visualized using the "maftools" R package (22). Venn diagrams identifying the mutations and amplified genes associated with OS in patients with LUSC were plotted by online tools (http://www.ehbio.com/test/venn/#/).

Identification of LUSC tumor antigens

To identify LUSC tumor antigens with higher immunogenicity, we analyzed the correlations of all potential tumor antigens with the level of infiltration of the 3 antigen-presenting cells using the tumor immune estimation resource (TIMER) online tool (23) (https://cistrome.shinyapps. io/timer/). Survival curves showing the expression of the potential tumor antigens were plotted using GEPIA 2 (24). In addition, the cohorts were divided into high and low groups using the expression of the 2 tumor antigens separately and analyzed for functional enrichment using the "gene set variation analysis (GSVA)" R package (25).

Identification of LUSC immune subtypes

All the immune-related genes were obtained from the

IMMPORT database (https://www.immport.org/shared/). Immune-related gene expression matrices from the TCGA-LUSC cohort were extracted and subjected to consensus clustering using the "ConsensusClusterPlus" R package. The optimal number of clusters was determined to be 5 using the elbow method. The HALLMARK gene set used for the gene set enrichment analysis (GSEA) of patients in the 5 clusters was obtained from the MSigDB database (http://www.gsea-msigdb.org/gsea/downloads.jsp).

Immune landscape analysis among clusters

Immune-stromal scores were calculated using the R package "ESTIMATE". The calculation of tumor purity was based on a previous publication (26). Tumor purity = cos (0.6049872018+0.0001467884× ESTIMATE score). A GSVA algorithm enrichment analysis was performed using 28 immune cell gene sets from previous publications (27). The immune checkpoints and immunogenic cell death modulators genes have been referred to in previously published articles (28).

Mutational landscape of LUSC immune subtypes

The tumor mutation burden (TMB) was calculated using the R package "maftools". The mutated driver genes were identified using MutsigCV software (29) and visualized using the R package "maftools".

Weighted gene co-expression network analysis (WGCNA)

The "WGCNAR" package (30) was used for the WGCNA with a soft threshold set at 50 to exclude outlying samples. In total, 6 gene co-expression modules were ultimately identified, and we calculated the correlations of the modules with the phenotypes of suitable vaccinated mRNA cancer vaccines from the above-mentioned immune landscape analysis. Finally, a univariate Cox regression analysis was performed of suitable vaccinations using genes in the most relevant modules to identify potential prognostic biomarkers after vaccination.

Drug sensitivity analysis

The drug sensitivity analysis was performed using the R package "pRRophetic" (31). In the analysis, we used the (genomics of drug sensitivity in cancer) GDSC database as the reference data to construct the model and performed an analysis of the sensitivity of multiple anti-cancer drugs on the 2 TCGA-LUSC subtypes that we had identified.

Statistical analysis

R software (version 4.1.1) (http://www.r-project.org/) and its corresponding R packages were used for statistical data analysis. Spearman correlation analysis was used to analyze the correlation between antigen-presenting cells and gene expression. A log-rank test was used to compare K-M curves for DFS and OS analysis. Kruskal-Wallis signed-rank test was used to compare gene expression, ESTIMATE scores, and TMB between multiple groups. Wilcoxon signed-rank test was used to compare the IC50 of anti-cancer drugs between two groups.

Results

Screening of potential tumor antigens in LUSC

To search for characteristic LUSC tumor antigens, we started with genomic variant events. First, regions of chromosomal copy number amplification in LUSC were identified by GISTIC software (see *Figure 1A*). We used all the copy number amplified genes as candidates for LUSC tumor antigens and plotted the chromosomal localization of partial genes (see *Figure 1B*). A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of all the copy number amplified genes showed their involvement in the regulation of cancer-related pathways and immune-related pathways (see *Figure 1C*). In addition, as genetic mutations play an important role in tumor development and progression, we also included all genes with mutations as candidates (see *Figure 1D*). All the LUSC tumor antigen candidates were intersected with genes related to patient survival obtained from the GEPIA database, and ultimately, 14 potential tumor antigens related to LUSC prognosis were obtained for the downstream analysis (see *Figure 1E*).

Identification of LUSC tumor antigens

The key mechanism of mRNA vaccines lies in the activation of adaptive immunity by recognition by antigen-presenting cells (9). Thus, we used the correlation between the expression of potential tumor antigens and the infiltration level of the 3 antigen-presenting cells to screen for potential mRNA vaccine antigens. Using the TIMER online tool
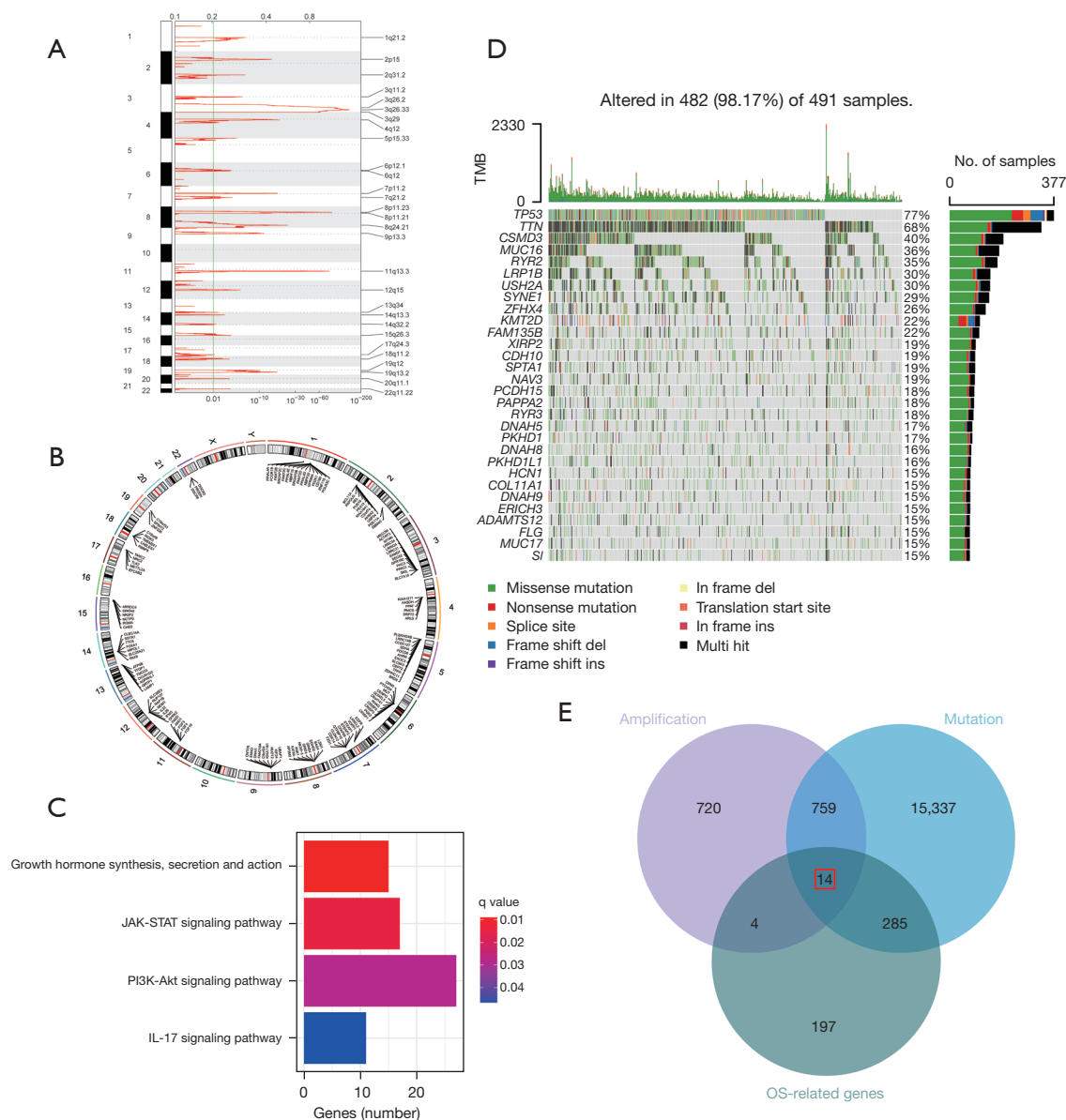
Figure 1 Identification of prognostic mutated genes in LUSC. (A) Significant copy number amplified genomic regions in LUSC identified by GISTIC2.0 Software. (B) Genomic mapping of genes with significantly amplified copy numbers. (C) KEGG functional enrichment analysis of all copy number amplified genes, x-axis is the number of enriched genes. (D) Gene mutation landscape in LUSC. (E) Identification of prognostically relevant mutated genes. TMB, tumor mutation burden; OS, overall survival; LUSC, lung squamous cell carcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes.

(https://cistrome.shinyapps.io/timer/), we identified 2 genes [i.e., bone morphogenetic protein 5 (BMP5) and claudin 5 (CLDN5)] in the 14 candidate tumor antigens whose expression presented a positive correlation with the level of infiltration of the antigen-presenting cells (see *Figure 2A,2B*). In addition, patients with high expression

of these genes had worse OS and disease-free survival (see *Figure 2C-2F*). After grouping patients according to the expression of these 2 genes, the GSEA-based KEGG pathway enrichment analysis showed that the high expression group of these 2 genes had enriched immune-related pathways, while the low expression group had a
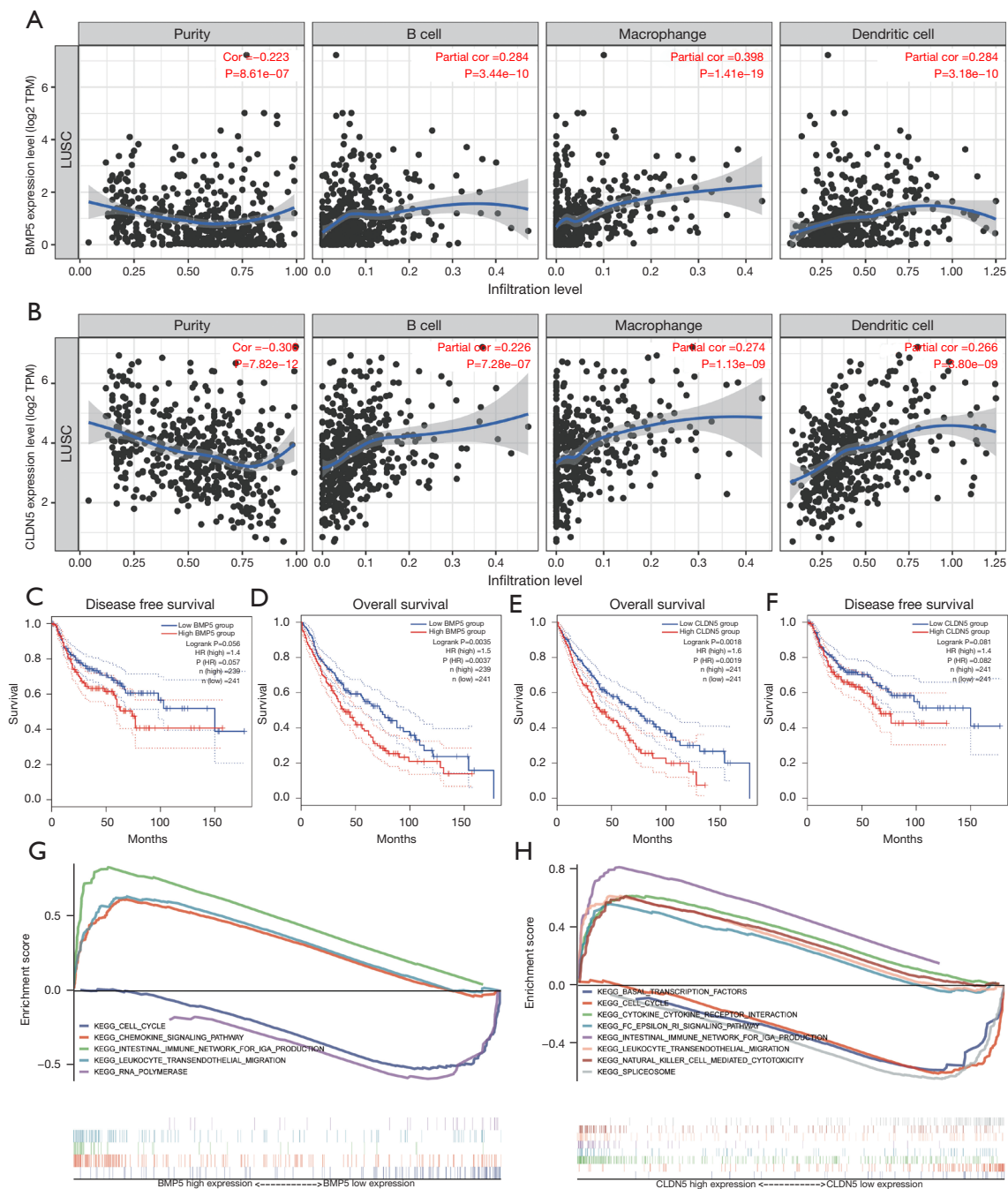
Figure 2 Identification of potential tumor antigens in LUSC. (A) Correlation of BMP5 expression with the infiltration of 3 antigen-presenting cells. (B) Correlation of CLDN5 expression with the infiltration of 3 antigen-presenting cells. (C) Disease-free survival curves after grouping TCGA-LUSC cohorts according to BMP5 expression. (D) OS curves after grouping TCGA-LUSC cohorts according to BMP5 expression (E) OS curves after grouping TCGA-LUSC cohorts according to CLDN5 expression (F) Disease-free survival curves after grouping TCGA-LUSC cohorts according to CLDN5 expression. (G) GSEA functional enrichment analysis of samples after BPM5 expression grouping. (H) GSEA functional enrichment analysis of samples after CLDN5 expression grouping. TPM, transcripts per kilobase of exon model per million mapped reads; cor, correlation; BMP5, bone morphogenetic protein 5; CLDN5, claudin 5; HR, hazard ratio; KEGG, Kyoto Encyclopedia of Genes and Genomes; LUSC, lung squamous cell carcinoma; TCGA, The Cancer Genome Atlas; OS, overall survival; GSEA, gene set enrichment analysis.

more active cell cycle (see *Figure 2G,2H*). Taken together with the above results, while the high expression of BMP5 and CLDN5 positively regulates immune-related pathways, the high expression of tumor cells is associated with a worse prognosis. Thus, mRNA vaccines developed based on these 2 genes to exogenously vaccinate patients can be recognized by antigen-presenting cells and promote tumor immunity, while avoiding the worse prognosis brought about by the high expression of the tumor cells themselves.

Immune subtypes in patients with lung adenocarcinoma

To identify a potentially beneficial population for mRNA vaccination, we used immune-related genes to identify the different immune statuses of patients with lung adenocarcinoma by consensus clustering. Using the elbow method, we ultimately identified 5 lung adenocarcinomas with different immune statuses (see *Figure 3A-3C*). In addition, a survival analysis of these patients with different immune statuses showed that Clusters C and D had a poor prognosis (see *Figure 3D*). To understand the tumor statuses of different patients, we performed an enrichment analysis of the HALLMARK gene set using the GSVA algorithm. The results showed that Cluster D was significantly enriched in multiple carcinogenic pathways, which may be responsible for the poor prognosis of cluster D (see *Figure 3E*). Finally, we also checked the expression of the 2 potential tumor antigens identified above that could be used for mRNA vaccine development. The results showed that BMP5 and CLDN5 were highly expressed in stage I and Cluster B, which suggests that mRNA vaccines developed based on our identified tumor antigens may have potentially better benefits for early stage and Cluster B patients than other patients (see *Figure 3F,3G*).

Immune cell infiltration landscape of different immune subtypes

To identify the immune landscape between immune subtypes, we evaluated the immune-stromal scores of each cluster using the ESTIMATE algorithm. In the results, the higher scores of immune and stromal cells in Clusters B, C, and D, and the lower tumor purity predicted a high level of immune cell infiltration in the tumor microenvironment (see *Figure 4A-4D*). To quantify different immune cells in the tumor microenvironment, we conducted a GSVA enrichment analysis using gene sets of 28 immune cells previously published (27). After comparing the immune cell

enrichment scores of different clusters, we also observed that Clusters B, C, and D had significantly higher immune cell infiltration than Clusters A and E (see *Figure 4E*). Thus, Clusters B, C, and D are immune "Hot" subtypes, while Cluster A and E are immune "Cold" subtypes (32), and patients in Clusters B, C and D may have better immunogenicity to mRNA vaccinations. In addition, we also compared the expression of immune checkpoints and immunogenic cell death modulator genes in each cluster (see *Figure 4F*). These genes were highly expressed in Clusters B, C, and D, which indicates that patients from these clusters may also have better sensitivity to immunotherapy, targeted therapy, or chemotherapy than other patients (see *Figure 4G,4H*). In conclusion, our findings reflect the diversity of tumor treatment for lung adenocarcinoma, and treatments should be precisely individualized for individuals to obtain better benefits.

Identification of mutation landscapes of different clusters

To identify the different mutational landscapes, we visualized the top 20 mutation genes of the TCGA-LUSC cohort and calculated the TMB for each sample (see *Figure 5A,5B*). We found that among all the clusters, only Cluster C had a lower TMB, which suggests that the development of a mutation-based gene-targeted therapy strategy for Cluster C will be limited, but the development of mRNA vaccines may provide a new therapeutic method for Cluster C. We also identified driver mutation genes in LUSC using the MutsigCV algorithm (29) and investigated the correlations among them (see *Figure 5C*). Overall, most of the mutations that occurred between them were independent, but there was a significant mutual exclusion of mutations in cyclin dependent kinase inhibitor 2A (CDKN2A) and retinoblastoma protein transcriptional corepressor 1 (RB1), while there was significant co-occurrence of mutations in neurofibromin 1 (NF1) and RAS P21 protein activator 1 (RASA1) (see *Figure 5D*).

WGCNA

To identify the genes associated with suitable vaccination clusters, we performed the module identification of all immune-related genes by WGCNA. The outlier samples were first sieved out by clustering the samples, and an optimal soft threshold of 8 was determined by connectivity (see *Figure 6A-6C*). Ultimately, we identified 5 modules in all the immune-related genes, and merged Clusters B, C, and
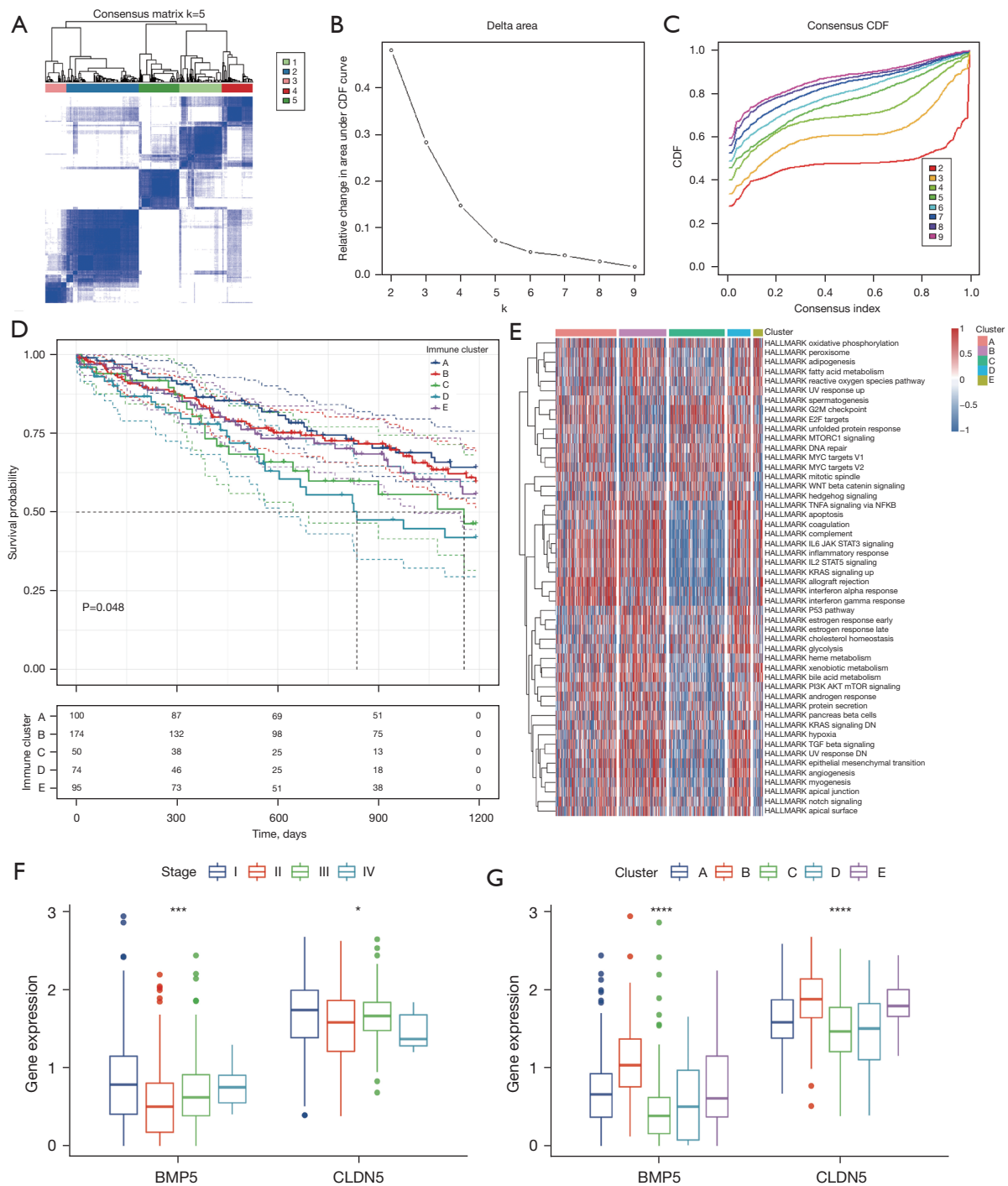
Figure 3 Identification of immune subtypes in patients with LUSC. (A-C) Consensus clustering of patients from the TCGA-LUSC cohort according to the expression of the immune-related genes. (D) Survival analysis of patients with different immune subtypes. (E) GSEA of the hallmark gene set in patients with different immune subtypes. (F) Expression of BMP5 and CLDN5 at different stages of LUSC. (G) Expression of BMP5 and CLDN5 in different immune subtypes. *, P value <0.05; ***, P value <0.001; ****, P value <0.0001. CDF, cumulative distribution function; BMP5, bone morphogenetic protein 5; CLDN5, claudin 5; LUSC, lung squamous cell carcinoma; TCGA, The Cancer Genome Atlas; GSEA, gene set enrichment analysis.

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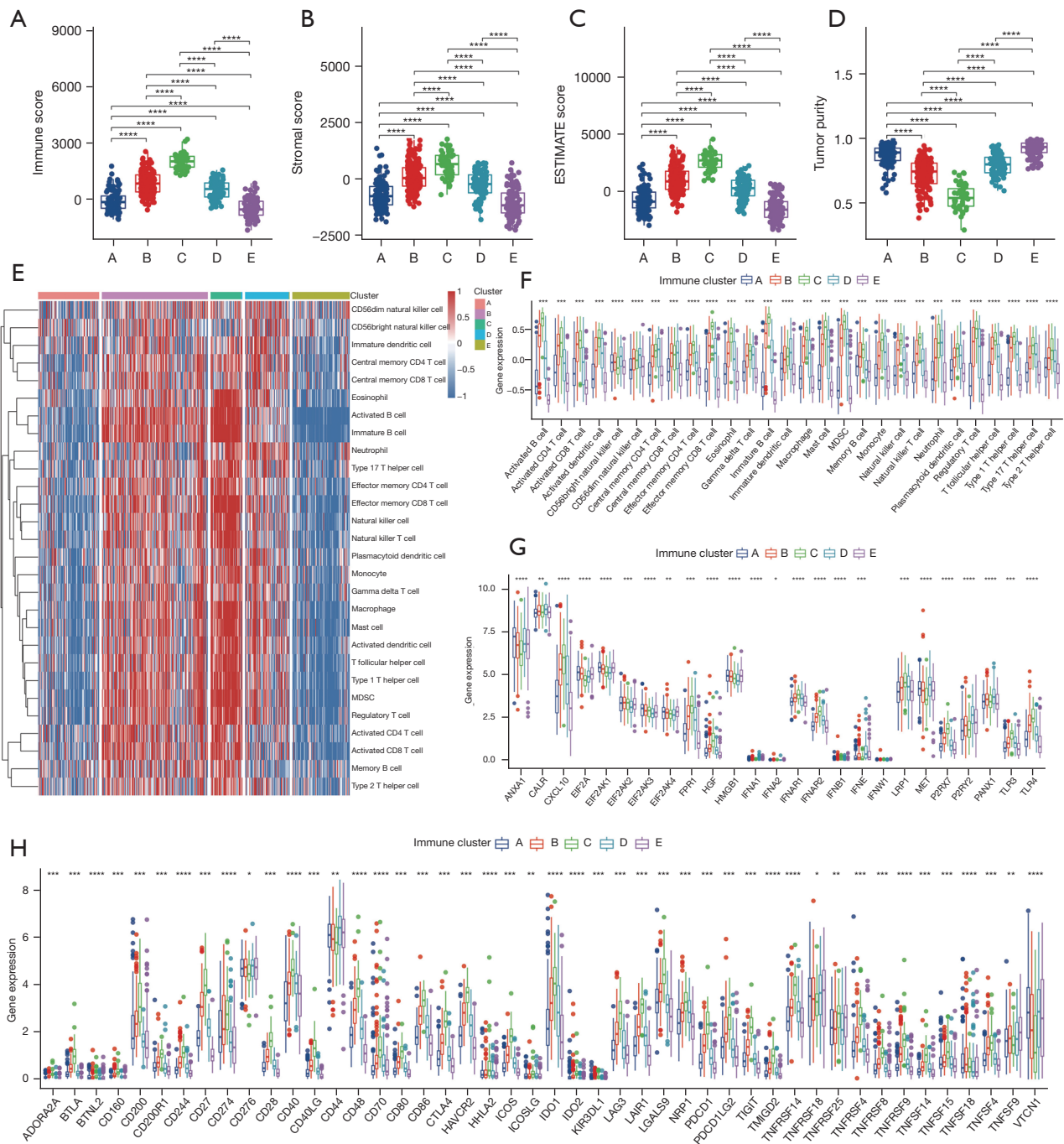
Figure 4 Immune landscape of different immune subtypes. (A-D) The estimate algorithm was used to calculate the immune-stromal scores for different immune subtypes. (E) Evaluation of immune cell infiltration in different immune subtypes using the GSVA algorithm. (F) Comparison of immune cell infiltration levels in different immune subtypes. (G) Immunogenic cell death modulates genes expression comparison between clusters. (H) Comparison of immune checkpoint expression between clusters. *, P value <0.05; **, P value <0.01; ***, P value <0.001; ****, P value <0.0001. GSVA, gene set variation analysis.
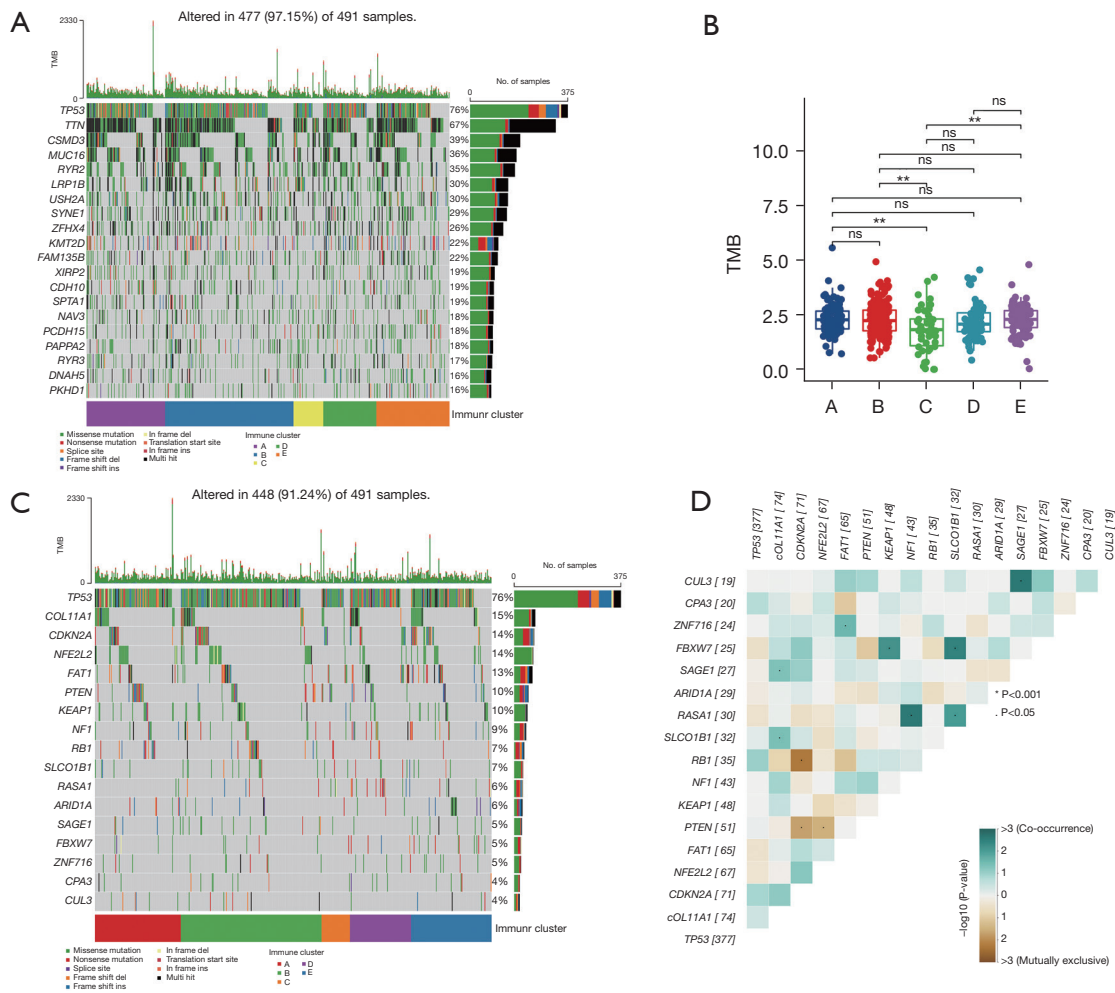
Figure 5 Mutational landscape of different immune subtypes in patients with LUSC. (A) Top 20 mutated genes in patients grouped according to immune subtypes. (B) Comparison of TMB in patients with different immune subtypes. (C) Driver mutations identified by the MutSigCV algorithm. (D) Co-mutations between the driver mutant genes. **, P value <0.01. ns, no significance; TMB, tumor mutation burden; LUSC, lung squamous cell carcinoma.

D as the "Suitable" group and Clusters A and E as the "Not-suitable" group. After calculating the association between the modules and phenotypes, we found that the population suitable for mRNA vaccination had the highest correlation coefficient with the "brown" module (see *Figure 6D,6E*). In addition, in a prognostic univariate Cox analysis in a suitable vaccinated population, we found 2 genes in "brown" module with P values <0.05 and a hazard ratio >1 [i.e., immunoglobulin heavy variable 7-81 (IGHV7-81) and immunoglobulin kappa variable 2-40 (IGKV2-40)] (see *Figure 6F*). These 2 genes were previously reported to be immunoglobulin component related genes (33). Thus, these 2 genes may be used as vaccine response biomarkers in LUSC patients suitable for mRNA vaccination.

Finally, we performed a drug sensitivity analysis of commonly used clinical treatments for samples suitable and unsuitable for mRNA vaccination and observed that patients unsuitable for mRNA vaccination were more sensitive to 2 chemotherapeutic agents (i.e., cisplatin, and etoposide) (see *Figure 6G,6H*). In addition, in relation to the current epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) commonly used in clinical practice, there was no significant difference in the sensitivity of gefitinib between the 2 groups, but there was a significant difference in the sensitivity of erlotinib between the 2 groups. We speculated that this may be due to the different EGFR mutation landscapes between the 2 groups (see *Figure 6I,6J*). Thus, the treatment of LUSC should focus on the
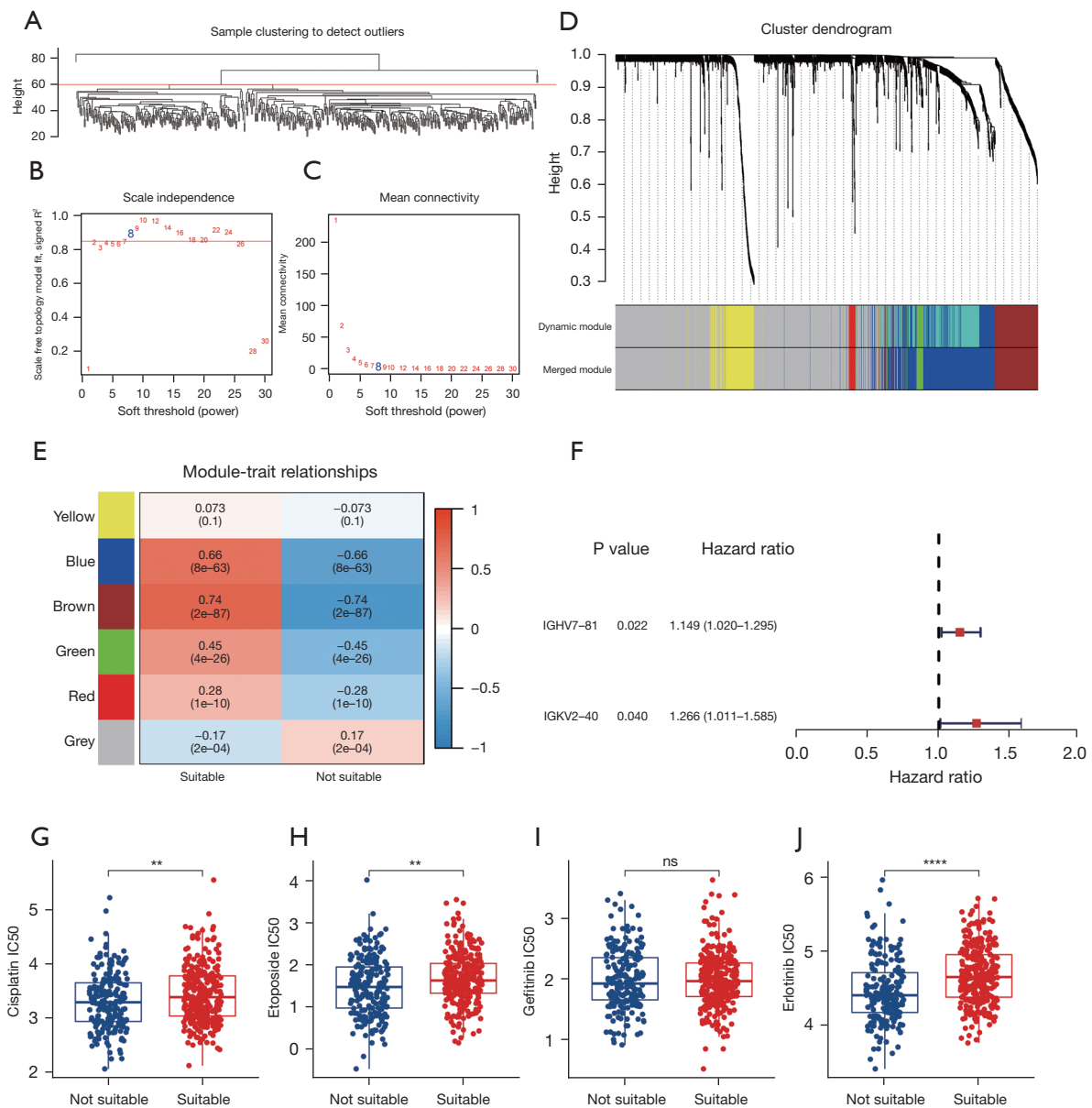
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Figure 6 Weighted gene co-expression network and drug sensitivity analyses. (A-E) The WGCNA revealed the association of gene modules with the phenotypes of suitable mRNA vaccinations. (F) The univariate Cox regression revealed potential monitoring biomarkers after mRNA vaccination in LUSC patients. (G-J) Anti-cancer drug sensitivity analysis of TCGA-LUSC. **, P value <0.01; ****, P value <0.0001. ns, no significance; WGCNA, weighted gene co-expression network analysis; Mrna, messenger ribonucleic acid; LUSC, lung squamous cell carcinoma; TCGA, The Cancer Genome Atlas.

development of an individualized treatment strategy.

Discussion

As an emerging immunotherapy, cancer vaccines have attracted much attention from researchers (6,9). In addition,

because mRNA vaccines do not integrate into the genome avoiding mutations generated during genome integration and can be degraded by abundant mRNA enzymes in the body, they are also easier to prepare than peptide vaccines (16,17,34). Thus, mRNA vaccines have potential clinical application value because of their easy preparation and long-

term safety (16,17,34). The main mechanism of action of cancer vaccines is that tumor antigens delivered by various carriers can be recognized by antigen-presenting cells and induce the body to produce immune memory to activate the immune system to produce anti-tumor effects (35,36). Thus, it is essential to search for tumor antigens with good specificity and immunogenicity for vaccine development in different cancers.

In this study, we screened mutant and copy number amplified genes associated with the prognosis of patients with LUSC to identify potential specific tumor antigen candidates for squamous cell carcinoma of the lung, and further analyzed their expression in correlation with the level of infiltration of 3 common antigen-presenting cells to determine their immunogenicity. Ultimately we identified 2 potential tumor antigens (i.e., BMP5 and CLDN5) that could be used for the development of mRNA vaccines for LUSC.

It has been reported that BMP5 is associated with clinical prognosis and plays a role as a tumor suppressor in patients with a variety of cancers (37-39). However, there are no relevant studies on BMP5 in LUSC. In our study, we found that patients with high BMP5 expression had a worse prognosis. Additionally, the activity of cell cycle-related pathways in patients in the high BMP5 expression group revealed the multiple role of BMP5 in cancer. In addition, BMP5 expression is associated with the infiltration of a variety of antigen-presenting cells in LUSC. Thus, due to its good immunogenicity, BMP5 can be used as a potential mRNA vaccine tumor antigen to block BMP5 targets by inducing immune memory.

In a previous study, CLDN5 was identified as a diagnostic biomarker for lung adenocarcinoma and LUSC (40). Additionally, CLDN5 can inhibit the cell cycle G1-S transition by decreasing the expression of cyclin D1 in LUSC cells (41). However, to date, no anti-tumor immune-related studies of CLDN5 in LUSC appear to have been conducted. Our study showed the good specificity and immunogenicity of CLDN5 as a potential LUSC tumor antigen, which could potentially be developed as a mRNA vaccine.

Individualized treatment is essential in overall cancer treatment (42,43), and different immune subtypes can reflect the current immune status of patients, and are informative for the prediction of treatment responsiveness (44,45). In this study, we clustered the entire TCGA-LUSC cohort using all the immune-related genes and identified 5 immune subtypes. A further analysis showed

that different immune subtypes had different survival states, different activation states of cancer-related pathways, and different mutational landscapes. Ultimately, we identified subtypes with abundant immune cell infiltration in the tumor microenvironment as potential markers of good responsiveness to mRNA vaccination, and further identified predictive potential benefits after vaccination by a WGCNA analysis. Through anti-cancer drug sensitivity analysis, we also found that patients who are not suitable for mRNA vaccine therapy may respond better to chemotherapy regimens based on Cisplatin, Etoposide and molecularly targeted drug regimens based on Erotinib.

Cancer vaccines are now considered to have potential clinical applications; however, there are still some problems to be solved in the development of vaccines (9). For example, the discovery of cancer antigens requires in-depth study and clinical validation by researchers, and the selection of vaccine vectors is related to the efficacy and long-term safety of cancer vaccines (46,47), and further studies are needed. However, the unique advantages of mRNA vaccines also make them an excellent prospect for application, and the development of LUSC mRNA vaccines based on BMP5, CLDN5 or other potential antigens will enrich the treatment options for LUSC patients.

Since our study is a bioinformatics-based study, the lack of experimental validation is a shortcoming of this paper. We have added a description in the discussion section to point out our shortcomings.

In conclusion, in this article, we identified 2 potential tumor antigens by bioinformatics methods that can be used for mRNA vaccine development in LUSC and analyzed the immune landscape of patients with LUSC, providing a new perspective for mRNA vaccine development in LUSC. There have been related studies on mRNA vaccine development in other cancers (28,48-52); however, this is the first study on mRNA vaccine development in LUSC. In addition, drug sensitivity analyses of populations with different immune subtypes also emphasize the importance of individualized treatment.

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Footnote

Reporting Checklist: The authors have completed the STREGA reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1113/rc

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1113/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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