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# Assessment of Neoantigen-Related Somatic Mutations in Non-Small Cell Lung Cancer

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**AUTHORS' CONTRIBUTION: (A)** Study Design · **(B)** Data Collection . **(C)** Statistical Analysis · **(D)** Data Interpretation · **(E)** Manuscript Preparation · **(F)** Literature Search · **(G)** No Fund Collection

ABSTRACT

**Aim:** The aim of this study is to examine the efficacy and safety of immune checkpoint inhibitors (ICIs) as a treatment for advanced non-small cell lung cancer (NSCLC). Neo-antigens are significant biomarkers and possible therapeutic targets that are crucial for the diagnosis and care of NSCLC patients. In materials from patients who had surgical therapy for NSCLC, this study sought to assess and define the connections between somatic mutations and possible neoantigens.

**Patients and methods:** This prospective study examined tissue samples from NSCLC patients who received surgery for their condition. Both tumour tissues and matched normal tissues underwent whole-exome sequencing. Using generative software, candidate neoantigens were predicted, and the associations between different mutational features and the quantity of neoantigens were assessed.

**Results:** Gene mutations connected to neoantigens occurred less frequently than mutations impacting the entire genome. High neoantigen burden genes exhibited a greater variety and frequency of mutations. There was a positive link between the number of putative neoantigens and missense mutations, code shift insertions/deletions, split-site variations, and mutations involving nonsense. However, only missense mutations show A>G/G>A and C>T/T>C base transitions and A>C/C>A, T>G/G>T, and C>G/G>C base transversions, respectively, had an inverse relationship with the number of neoantigens.d a positive correlation with the quantity of neoantigens in the analysis of multiple linear regression.

**Conclusion:** The frequency, kind, and base substitution type of mutations were all linked to the quantity of potential neoantigens found in NSCLC cases.

**Keywords:** Non-small cell lung cancer; Whole exome sequencing; Neoantigens; Tumor neoantigen burden; Genetic mutation characteristics

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## INTRODUCTION

Despite improvements in treatment methods over the past 20 years, lung cancer continues to be the top reason for cancer-related deaths globally. Immune checkpoint inhibitors (ICIs) are compounds produced from antibodies that have only lately begun to be used as cancer therapeutic options. These therapies have significantly improved clinical outcomes and altered the landscape of therapy options for people with stage IV non-small cell lung cancer (NSCLC). Despite the fact that chemotherapy is less effective than ICIs in first- and second-line treatments, the objective response rate among unselected patients is only about 20%. [1–11]. They target regulatory receptors like cytotoxic T lymphocyte associated protein 4 (CTLA-4), programmed cell death-ligand 1, and regulated cell death-1 (PD-1) (PD-L1). Therefore, it's critical to carefully choose patients who are anticipated to gain from ICI treatment. PD-L1 expression is the only currently recognized biomarker for estimating ICI therapy response. However, patients with minimal tumour PD-L1 expression can still benefit from treatment, indicating that PD-L1 is not always reliable for identifying candidates for immunotherapy [12]. Major Histocompatibility Complex (MHC) expression, lymphocyte count, tumour T-cell marker expression, tumour burden (TMB), and neoantigen expression are additional possible biomarkers for ICI treatment guidance [13]. Neoantigens produced by mutations have garnered a lot of interest among possible biomarkers. These mutant peptides that are unique to tumour cells can be presented by MHC molecules and identified by T lymphocytes. Neoantigens can therefore mediate the immune response to tumour cells and enable the host immune system to identify and eliminate them [14, 15]. Recent developments in genomics and bio-informatics have set the stage for the efficient selection of the most immunogenic neoantigens from the range of somatic mutations present in tumours. However, there aren't many data on gene alterations linked to neoantigens in NSCLC. Somatic mutations and possible neoantigens were examined in specimens from individuals who underwent surgical therapy for NSCLC in order to assess and characterize this association. Using these data, it may be feasible to identify patients who would benefit from ICI treatment.

## PARTICIPANTS AND METHODS

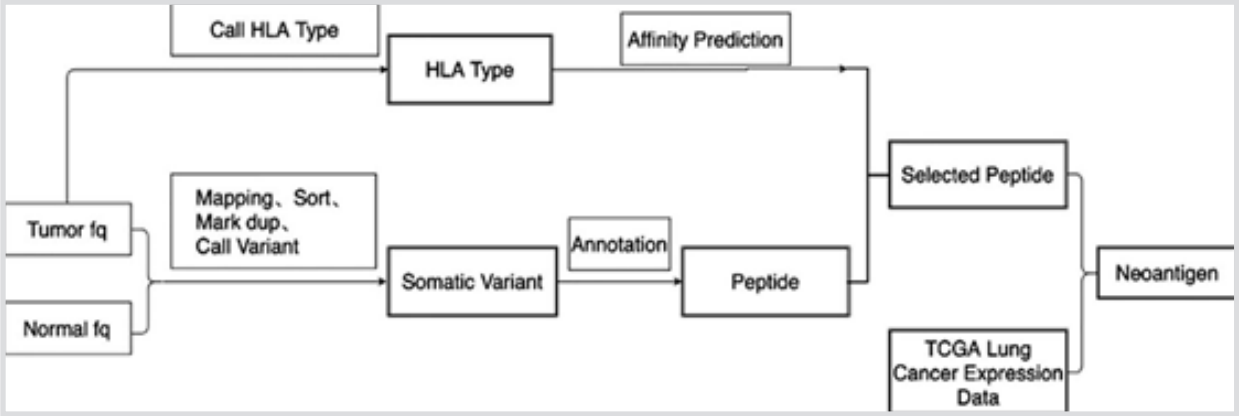
### Participants

We prospectively gathered NSCLC patients who underwent

surgical therapy at the thoracic surgery department between June 2019 and September 2019. The inclusion criteria included Histopathologically confirmed NSCLC, thorough clinical and pathological information, enough tumour and matched normal tissue for whole-exome sequencing, and patient agreement to the use of their tissues for study. Sex, age, smoking history, histological type, TNM stage, and clinical stage were all recorded. The protocol for the study received clearance from our institutional review board.

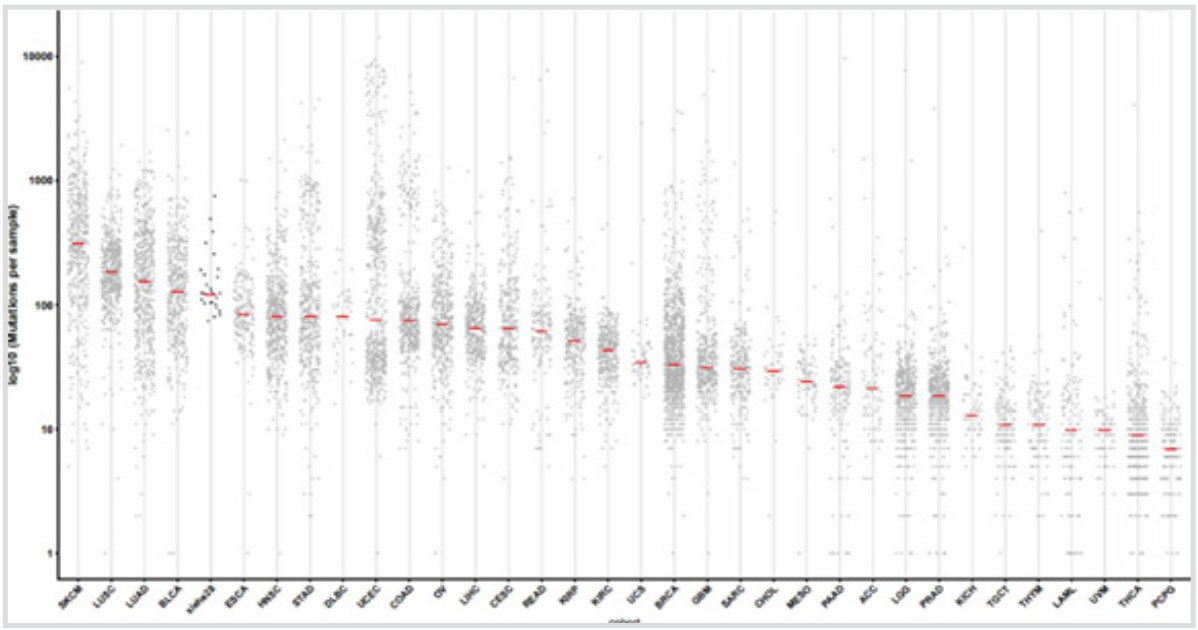
## Neoantigen prediction

Results from whole-exome sequencing were obtained for both the matched normal tissues and malignant tissues. Neopipe software was then used to identify potential neoantigens by correlating gene expression with the molecule's anticipated affinity for class I MHC **(Fig.1.).** Data on RNA expression could not be collected due to financial constraints. However, gene expressions for NSCLC were sourced from the TCGA database **(Fig.2.).**




1) The original tumour and normal FASTQ data were subjected to quality control and statistical analysis. 2) FASTQ data were compared to the human reference genome HG19 using Bwa. The readings were marked for duplication using Samblaster. 3) SamTools was used to generate the index and convert the Sam file to a BAM file. 4) To identify somatic mutations, filter them using GATK Mutect2, and then collect mutation data. 5) Using Optitype, the original FASTQ data was used to type HLA. 6)Netmhcpan4 was employed to forecast peptide affinity based on HLA type.7) The mean values of the expression data for patients with lung adenocarcinoma and lung squamous cell carcinoma were determined from the lung cancer patients' TCGA data, which were retrieved. 8) To determine the final neoantigen results, neoantigen scores were calculated based on the sequencing data, mutation frequency, HLA, transcript, and immune-related gene expression.

**Fig.1**. Flow chart of candidate neoantigen design.




TCGA database. The log 10 (mutations per sample) scale is shown on the Y-axis. The figure's red line denotes the mean value. Skin cutaneous melanoma is to be noted. lung squamous cell carcinoma, or LUSC. Lung adenocarcinoma, or LUAD. HNSC, head and neck squamous cell carcinoma; ESCA, esophageal cancer; BLCA, bladder cancer; colon adenocarcinoma; uterine corpus endometrial carcinoma; diffuse large B-cell lymphoma; stomach adenocarcinoma; BRCA, breast cancer; GBM, glioblastoma; SARC, sarcoma; CHOL, cholangiocarcinoma; MESO, mesothelioma; OV, ovarian cancer; LIHC, liver hepatocellular carcinoma; CESC, cervical squamous cell carcinoma; READ, rectal adenocarcinoma; KIPP, kidney renal papillary cell carcinoma; KIRC, kidney renal clear cell carcinoma; UCS, uterine carcino-sarcoma; BRCA, breast cancer; GBM, glioblastoma; SARC, sarcoma; CHOL, cholangiocarcinoma; MESO, mesothelioma; PAAD, pancreatic adenocarcinoma; ACC, adrenocortical carcinoma; LGG, lower grade glioma; PRAD, Prostate adenocarcinoma; KICH, kidney chromophobe; TGCT, tenosynovial giant cell tumour; THYM, thymoma; LAML, acute myeloid leukaemia; UVM, uveal melanoma; THCA, thyroid carcinoma; PCPG, pheochromocytoma and paraganglioma.

**Fig.2.** Map of gene disruption.

574 lung adenocarcinoma cases and 548 lung squamous cell carcinoma cases, respectively, were used to collect the mean values for transcriptional and genetic quantification for lung adenocarcinoma and lung squamous cell carcinoma. The average transcriptional and genomic quantification values for all other disease categories were gathered from all NSCLC cases.

Neoantigens were created as 25-mer peptides to make future study on in vitro production and delivery easier. Neoepitopes are 8–11–mer mutated peptides that can bind to MHC. The accuracy of forecasting immune stimulation would be limited if it was based solely on the quantity of neoantigen epitopes due to the variation in neoantigen epitopes and MHC affinities. Therefore, without taking into account neoepitopes, the key results included the characteristics of gene mutations connected to neoantigens.

## Statistical analysis

The SPSS programme (version 21.0; IBM Corp., Armonk, NY, USA) was used for all analyses. The right metrics are used to present the results, including median, number, frequency, and composition ratio. Non-parametric testing was used to analyse clinicopathological features related to the number of neoantigens. The Spearman's test was used to investigate the connection between neoantigens and the characteristics of gene mutations. Multiple linear regression analysis was performed for the various neoantigen-related gene mutation types. Using the R studio programme, heat maps were produced, and related cluster analyses were carried out. Results were deemed statistically significant using a bilateral test when $P < 0.05$.

# RESULTS

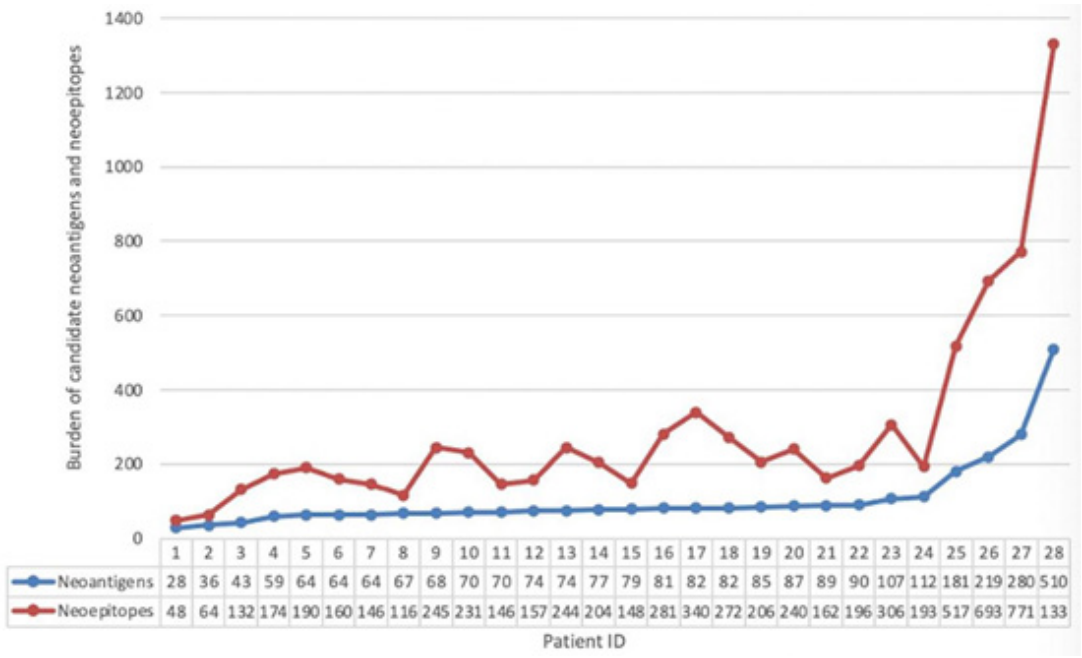
## Clinicopathological features and prediction of candidate neoantigens

34 patients received surgery for NSCLC at our facility between June and September of this year. Due to insufficient tissue samples or missing clinical data, we had to eliminate 6 cases. 28 patients were subsequently included in the trial as a result. The range of ages was 38 to 76, with a median age of 60.5 years. Fifteen patients (53.6%) had a history of smoking. seventeen patients (60.7%) were male. The pathological classifications were large-cell neuroendocrine carcinoma (1 case, 3.6%), squamous cell carcinoma (3 instances, 10.7%), and adenocarcinoma (24 cases, 85.7%). (19 individuals or 67.9% were in stage I) Stages II and III each had six individuals (21.4% and 10.7%, respectively) were used to categorize the tumours. Six patients (21.4%) have a history of malignancies in their family **(Tab.1.).**

Whole-exome sequencing of 28 NSCLC cases yielded a total of 5,017 non-synonymous variants, of which 4,037 were missense mutations, 419 were frame-shift insertions/deletions, 313 were in-frame insertions/deletions, 229 were nonsense mutations, 10 were non-stop mutations, and 9 were splice site mutations. The 7,452 single-nucleotide variants included A>T/T>A (n=539), A>C/C>A (n=966), A>G/G>A (n=2,006), T>C/C>T (n=1,990), T>G/G>T (n=1,025), and C>G/G>C (n=926). Based on the results from the 28 specimens, the Neopipe programme projected a total of 2,942 neoantigens (median: 78, range: 28-510) and 7,912 neoepitopes (median: 200, range: 48-1,300) **(Fig.3.).** The largest tumour diameter and the number

**Tab. 1.** Clinicopathological characteristics of 28 patients.

| No. | Gender | Age | Smoking history (pack years) | Pathology | TNM stage | Clinical stage | Tumor size | Tumor history |
|---|---|---|---|---|---|---|---|---|
| 1 | Male | 38 | No | A | T1bNOMO | Ia2 | 14mm | No |
| 2 | Male | 61 | 20 | A | T1bNOMO | Ia2 | 23mm | No |
| 3 | Male | 59 | No | A | T1aNOMO | Ia1 | 10mm | No |
| 4 | Male | 58 | 30 | A | T1aNOMO | Ia1 | 8mm | No |
| 5 | Female | 70 | No | A | T1bNOMO | Ia2 | 20mm | No |
| 6 | Female | 76 | No | A | T1bNOMO | Ia2 | 20mm | Yes |
| 7 | Male | 56 | 30 | A | T1bN1MO | IIb | 20mm | No |
| 8 | Male | 70 | 10 | A | T2aNOMO | Ib | 40mm | No |
| 9 | Male | 59 | No | A | T1cNOMO | Ia3 | 25mm | No |
| 10 | Male | 47 | 30 | A | T2bN2MO | IIIa | 20mm | No |
| 11 | Female | 52 | No | A | T1aNOMO | Ia1 | 10mm | No |
| 12 | Female | 41 | 2 | A | T3N2MO | IIIb | 20mm | No |
| 13 | Female | 60 | No | A | T1cNOMO | Ia3 | 18mm | No |
| 14 | Female | 68 | No | A | T1bNOMO | Ia2 | 15mm | No |
| 15 | Male | 73 | No | A | T1cNOMO | Ia3 | 30mm | Yes |
| 16 | Female | 60 | No | A | T1bNOMO | Ia2 | 13mm | No |
| 17 | Female | 54 | 3 | A | T1cNOMO | Ia3 | 27mm | Yes |
| 18 | Female | 71 | No | A | T1bNOMO | Ia2 | 15mm | No |
| 19 | Female | 58 | No | A | T3NOMO | IIb | 60mm | No |
| 20 | Female | 63 | No | A | T1bNOMO | Ia2 | 20mm | No |
| 21 | Male | 49 | 30 | S | T2aN1M0 | IIb | 32mm | No |
| 22 | Male | 61 | 40 | A | T1bNOMO | Ia2 | 15mm | Yes |
| 23 | Male | 61 | 35 | A | T2bN1M0 | IIb | 40mm | No |
| 24 | Male | 56 | 30 | A | T2bN2MO | IIIa | 28mm | No |
| 25 | Male | 63 | 40 | A | T2aNOMO | Ib | 36mm | Yes |
| 26 | Male | 64 | 50 | S | T1cN1M0 | IIb | 30mm | No |
| 27 | Male | 64 | 3 | S | T2bN1MO | IIb | 60mm | Yes |
| 28 | Male | 64 | 40 | LCNEC | T2aNOMO | Ib | 40mm | No |




Nine patients out of the total of twenty-eight exhibited EGFR-sensitive mutations, including six cases of 21L858R, two cases of 19DEL, and one case of 20INS. According to non-parametric analysis, there was no correlation between the quantity of potential neoantigens and EGFR mutations (P = 0.087). (Table S1). One patient had a ROS1 fusion and another had an EML4-ALK fusion (85 potential neoantigens) (36 candidate neoantigens). Three patients each possessed one of the following KRAS mutations: KRAS G12D (87 neoantigens), KRAS G12D paired with CDKN2A D108H (28 neoantigens), or KRAS G12V combined with TP53 K132E and STK11 N181Y mutations (85 neoantigens).

**FIGURE 3:** Neo-epitope and neoantigen maps of the patients included in our study.

of anticipated neoantigens were found to be positively correlated by Spearman's correlation analysis (correlation coefficient=0.575, P=0.001). Additionally, patients with squamous cell carcinoma (rank mean: 26.47 vs. 13.28, P=0.019) and patients with a family history of tumours had a larger number of potential neoantigens (rank mean: 20.42 vs. 12.89, P=0.046).

## Characteristics of gene mutations associated with candidate neoantigens

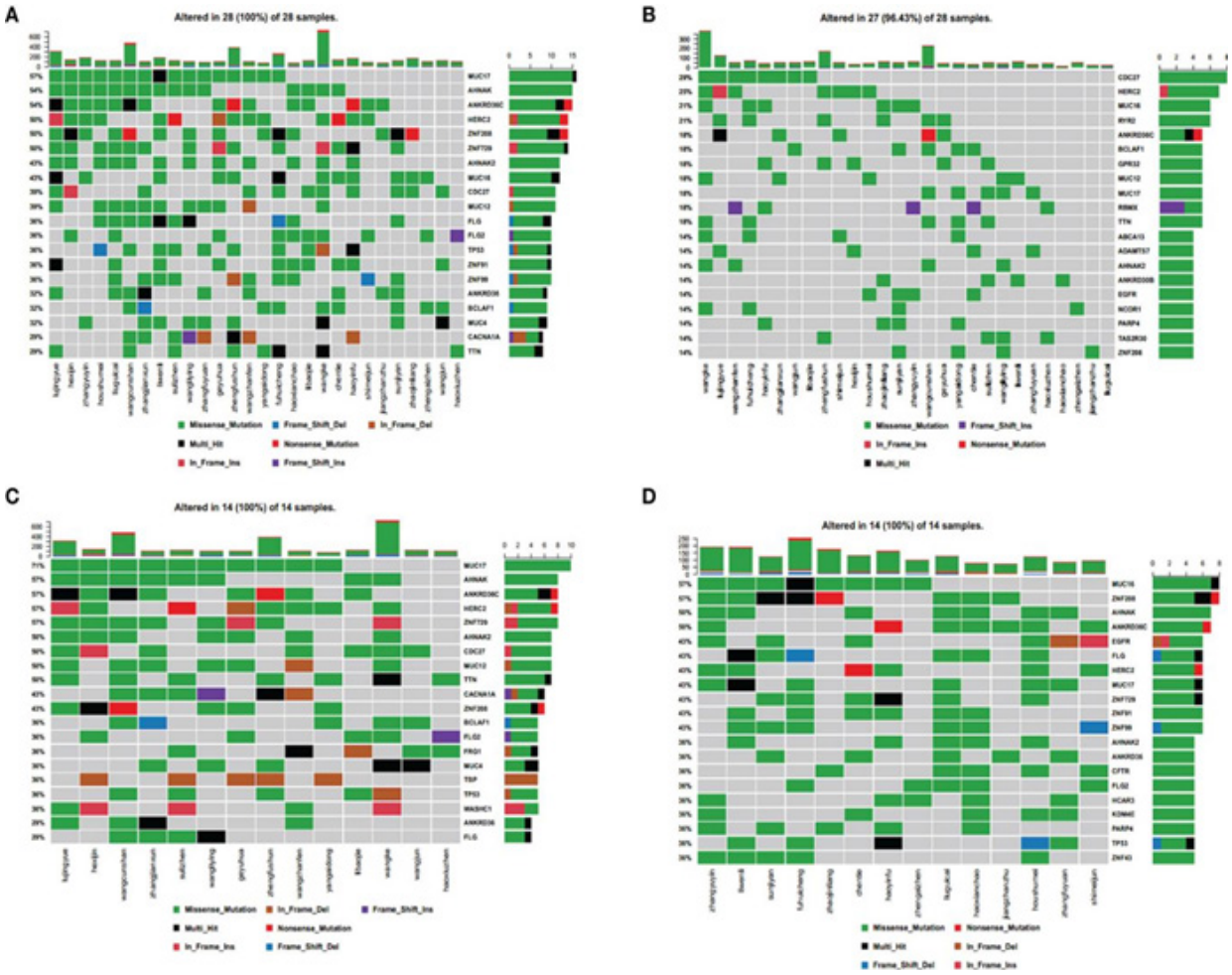
The investigation of whole genome mutations and neoantigen-related gene modifications revealed that the ten most frequently mutated genes were MUC17 (57%), AHNAK (54%), ANKRD36C (54%), HERC2 (50%), ZNF208 (50%), ZNF729 (50%), AHNAK2 (43%), MUC16 (43%), CDC27 (39%), and MUC12 (39%) **(Fig. 4A.).** The 10 neoantigen-related genes that received the most mutations were CDC27 (29%), HERC2 (25%), MUC16 (21%), ANKRD36C (21%), BCLAF1 (18%), GPR32 (18%), MUC12 (18%), MUC17 (18%), PBMX (18%), and TTN (18%) **(Fig. 4B.).** The whole genome mutations had higher frequencies and a wider range of alterations, including missense mutations, nonsense mutations, in-frame deletions, frame-shift deletions, in-frame insertions, and mixed mutations. On the other hand, only missense, nonsense, in-frame, frame-shift, and mixed mutations were the most prevalent types of neoantigen-related gene alterations.

## Neoantigen load-related mutation characteristics comparison

Cancer neoantigen burden (TNB) is the total number of neoantigens per million bases (Mbs) in a cancer sample. In order to compare the traits of the gene mutation, patients were split into two groups depending on their median TNB value (n=14), a high TNB group (n>14) and a low TNB group (n 14). The 10 most commonly changed genes in the high TNB group were MUC17 (17%), AHNAK (57%), ANKRD36C (57%), HERC2 (57%), ZNF729 (57%), AHNAK2 (50%), CDC27 (50%), MUC12 (50%), TTN (50%), and CACNA1A (43%) **(Fig. 4C.).** The 10 most commonly changed genes in the low TNB group were MUC16 (57%), ZNF208 (57%), AHNAK (50%), ANKRD36C (50%), EGFR (43%), FLG (43%), HERC2 (43%), MUC17 (43%), ZNF729 (43%), and ZNF91 (43%) **(Fig. 4D).** Other mutation types, including mixed mutations, were more common in the genes of patients with high TNB, as were missense mutations, nonsense mutations, in-frame deletions, frame-shift deletions, in-frame insertions, and mixed mutations. Furthermore, individuals with high TNB were much more likely to have mixed mutations, insertion mutations, and deletion mutations than patients with low TNB. Patients with low TNB had missense mutations, nonsense mutations, in-frame deletions, frame-shift deletions, in-frame insertions, and mixed mutations. Neoantigen burden (TNB) is thereby correlated with both the quantity and type of gene mutations, with neoantigen-related mutations having fewer mutation types than whole genome mutations. Patients with a high TNB also had a greater variety of gene mutations than patients with a low TNB, indicating that some mutation types are more common than others.

## Several gene mutation patterns' implications on potential neoantigens

To access the characteristics of neoantigen-related gene




(A) All mutations; (B) mutations connected to neoantigens. Common gene mutations in patients are shown in a spectrum heat map. (C) With a high burden of tumour neoantigens; (D) with a low load of tumour neoantigens.

**FIGURE 4:** Heat map of common gene mutations in spectrum form.

mutations, Spearman's correlation analysis was carried out **(Tab. 2.).** The frequency of non-synonymous mutations and the number of neoantigens were strongly associated (correlation coefficient=0.641, P <0.001). Following annotation and analysis of all non-synonymous mutation types, neoantigen abundance was found to be strongly linked with missense mutations (correlation coefficient = 0.603, P <0.001), frame-shift insertions/deletions (correlation coefficient=0.755, P <0.001), nonsense mutations (correlation coefficient=0.501, P =0.007), and splice site mutations (correlation coefficient=0.546, P =0.003) **(Tab. 2.).** In a multiple linear regression analysis that included these four mutation types, only missense mutations (beta=0.674, P <0.001) were positively linked with the neoantigen quantity **(Tab. 3.).** This might be connected to how frequently these mutations occur. Furthermore, despite the fact that they may result in a higher neoantigen burden, the lack of statistical significance between the quantity of potential neoantigens and other mutation types may be due to their rarity. Additionally, Spearman's correlation analysis was used to examine the relationship between base substitution and the quantity of neoantigens. The following base transversions showed a positive correlation with the frequency of neoantigens: (Correlation coefficient = 0.641, A>C/C>A), P <0.001), T>G/G>T (correlation coefficient=0.388, P=0.041), and G>C/C>G (correlation coefficient=0.418, P=0.027). Base transitions such as A>G/G>A had a negative connection with the quantity of neoantigens (correlation coefficient=-0.690, P<0.001) and T>C/C>T (correlation coefficient=-0.535, P=0.003) **(Tab. 2.).**

As a result, the number of putative neoantigens was associated with both the quantity and kind of mutations found in tumours, as well as with base transversions and base transitions.

## Multiple candidate neoantigens associated with gene mutations

Neoantigens were associated with 1,922 gene alterations, and 1–28 neoantigens were produced by each modification. There were 21 genes among them that were connected to seven neoantigens. When these 21 genes underwent cluster analysis, there was no discernible relationship between the quantity of neoantigens and the expression of those genes in each patient.

# DISCUSSION

When treating NSCLC patients with elevated TMB, ICIs are more successful. This has given rise to the

**Tab.2.** Candidate neoantigen Spearman correlation analysis.

| Neoantigens | N | Correlation coefficient | P-value |
|---|---|---|---|
| nonsynonymous mutation | 28 | 0664 | <0.001 |
| Frame shift indel | 28 | 0.755 | <0.001 |
| In frame indel | 28 | 0.071 | 0.718 |
| Missense mutation | 28 | 0.603 | 0.001 |
| Nonsense mutation | 28 | 0.501 | 0.007 |
| Nonstop mutation | 28 | 0.211 | 0.282 |
| Splice site | 28 | 0.546 | 0.003 |
| A>T/T>A mutation frequency | 28 | 0.279 | 0.151 |
| A>C/C>A mutation frequency | 28 | 0.641 | <0.001 |
| A>G/G>A mutation frequency | 28 | -0.690 | <0.001 |
| T>C/C>T mutation frequency | 28 | -0.535 | 0.003 |
| T>G/G>T mutation frequency | 28 | 0.388 | 0.041 |
| C>G/G>C mutation frequency | 28 | 0.418 | 0.027 |

**Tab.3.** Prospective neoantigens with multiple linear regression analysis.

| Variants | Unstandardized coefficients | | Unstandardized coefficients Beta | t-value | P-value | 95.0% confidence interval of B | |
|---|---|---|---|---|---|---|---|
| | B | Standard error | | | | Lower limit | Higher limit |
| (Constant) | -2.811 | 10.84 | | -0.259 | 0.798 | -25.235 | 19.614 |
| Frameshift indel | 1.612 | 1.139 | 0.154 | 1.415 | 0.17 | -0.744 | 3.968 |
| Missense mutation | 0.5 | 0.115 | 0.674 | 4.342 | 0 | 0.262 | 0.739 |
| Nonsense mutation | 0.782 | 1.418 | 0.08 | 0.552 | 0.587 | -2.152 | 3.716 |
| Splice site | 16.209 | 12.101 | 0.104 | 1.339 | 0.194 | -8.825 | 41.243 |

hypothesis that TMB may be a biomarker for determining how well ICI treatment will work [16]. Furthermore, preclinical and clinical investigations have suggested that certain neoantigens produced by tumor-specific missense mutations may regulate the response to ICIs [17]. This implies that a high TMB could result in the development of more neoantigens, which would boost immunogenicity and improve response to ICI treatment [18]. Our research, which revealed a positive correlation between non-synonymous mutations and the quantity of neoantigens, provides additional evidence for this. The number of neoantigens and missense mutations, the most common mutation type, as well as less common mutation types including frame-shift insertions/deletions, nonsense mutations, and split-site mutations, were significantly correlated, according to other classifications and analyses. There is proof that non-synonymous single nucleotide mutations occur more frequently than frame-shift insertions or deletions. They may, however, be highly immunogenic alterations that boost neoantigen burden and increase MHC affinity [19, 20]. Numerous studies have suggested that splicing that is particular to tumours is a significant source of neoantigens [21-23]. Despite the low frequency of splicing, splicing sites produce neoantigens more frequently than single-nucleotide mutations do [24]. We discovered that nonsense mutations occurred less frequently.

However, it also showed a positive correlation with the quantity of neoantigens, suggesting that nonsense mutations can result in a higher concentration of neoantigens. To the best of our knowledge, no research on the relationship between nonsense mutations and neoantigens has been published; hence, more research is required to address this problem. Additionally, we noted a positive correlation between base transversions and the neoantigen burden and a negative correlation between base transitions. According to a previous study [25] of patients who received pembrolizumab, individuals with a durable therapeutic benefit were more likely to have C>A transversions and less likely to have C>T transitions (Mann-Whitney test; P=0.01). These outcomes are in line with what we have seen.

In earlier investigations, 9,155 tumour samples from the International Cancer Genome Consortium database were utilised to predict possible neoantigens from somatic mutations using the TSNAD software. They showed that KRAS, PIK3CA, and TP53 encoded the most prevalent possible neoantigens. For instance, six of the ten most common potential neoantigens were from the KRAS gene and involved the G12D and G12V mutations [26]. In a distinct examination of genomic, transcriptomic, and proteomic data from KRAS-mutated lung adenocarcinoma, three biological subgroups were identified: STK11/LKB1 (KL subtype), TP53 (KP subtype), and CDKN2A/B (KC subtype). Lung adenocarcinoma of the KP subtype displayed a marked inflammatory response and increased expression of numerous co-simulators and co-suppressors in this situation.

In contrast, immunological markers were expressed at lower levels in lung adenocarcinomas of the KL subtype. The KP subtype has a higher mutation rate than the KL subtype despite having experienced comparable smoking exposures, which may account for the variations in their immunogenicity [27]. In our analysis, there were only three KRAS mutations among the twenty-eight patients, one of which had a KRAS G12V mutation (a KP and KL mixed type) and 85 potential neoantigens.In a patient with a KRAS G12D mutation (the KC subtype), 28 candidate neoantigens were discovered, whereas 87 candidate neoantigens were discovered in a patient with just a KRAS G12D mutation (no combination mutations).

## CONCLUSION

Only the patient with the KC subtype had a significantly lower number of potential neoantigens than the median value; the numbers in the other two individuals were just marginally higher. As a result, our findings do not agree with those that have been previously reported about the relationship between KRAS mutations and neoantigens.
The extremely small sample size used in our analysis may help to explain this (only three patients with KRAS mutations). Our study has two significant flaws. First of all, because to funding limitations, we were unable to obtain RNA-related data to aid in neoantigen prediction. Instead, this was based on the TCGA database's expression data for genes connected to lung cancer. Second, a larger study will be required to confirm our findings because the sample size was modest.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee at the Lugansk State Medical University and Hospital. The patients/participants provided their written informed consent to participate in this study.

## CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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