Original paper

The impact of *KRAS* mutations on the tumour microenvironment and treatment response in non-small cell lung cancer

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Mutations in the *KRAS* gene in non-small cell lung cancer (NSCLC) are common drivers. Gene expression and mutation data of NSCLC were collected from the TCGA dataset. DEGs between *KRAS* mutations and wild type were identified, and enrichment analysis was performed. The differences in immune cell infiltration between the 2 groups were evaluated using ssGSEA, and TIDE scoring, immune checkpoint therapy sensitivity, and drug treatment sensitivity analysis were performed. The expression of PD-L1 and CTLA-4 in tumour tissues was detected by western blot. CD8+PD-1 and CD8+CTLA-4 cells were detected by flow cytometry. The frequencies of *KRAS*-G12C, *KRAS*-G12V, and *KRAS*-G12D mutations were the highest. A total of 1323 DEGs were predominantly enriched in the PI3K-Akt signalling pathway, cell adhesion molecules, and metabolism of xenobiotics by cytochrome P450. Additionally, most immune cell infiltration levels in *KRAS* mutations were lower than in *KRAS* wild type. Sensitivity to immune checkpoint inhibitors and drug treatments increased in *KRAS* mutations. Western blot revealed significantly higher expressions of PD-L1 and CTLA-4 in *KRAS* mutations compared to *KRAS* wild type. The infiltration of CD8+PD-1+ T cells and CD8+CTLA-4+ T cells was higher in *KRAS* mutations than in *KRAS* wild type. *KRAS*-G12C, *KRAS*-G12V, and *KRAS*-G12D mutations may enhance NSCLC drug resistance through immunosuppression.

Key words: non-small cell lung cancer, *KRAS*, immune checkpoint, treatment response.

Introduction

Lung cancer is the leading cause of cancer deaths worldwide [1]. Despite the implementation of screening programs, which are expected to promote early detection and improve outcomes, nearly 50% of lung cancers are diagnosed at advanced and metastatic stages [2]. Approximately 85% of lung cancers are non-small cell lung cancer (NSCLC), with the 5-year survival rate for metastatic patients being only 4%, resulting in significant social burden and economic loss [3]. The prognosis of NSCLC is extremely challenging due to the late appearance of symptoms during the disease's progression, limiting treatment options and survival rates.

With the increase of anti-tumour cells such as CD8+ T cells, immune cells are activated in the early stages but quickly suppressed in the later stages [4]. Multiple immune escape mechanisms have been reported in NSCLC, including the exploitation of immune checkpoints to facilitate immune escape [5]. Immune checkpoint inhibitors (ICIs) such as those targeting the PD-L1/PD-1 pathway and CTLA-4 disrupt tumour cells and re-activate effector T cells

leading to tumour regression [6]. Significant advancements in targeted therapy and immunotherapy have altered the treatment modalities and survival outcomes for NSCLC patients [7]. However, treatment resistance remains a long-term challenge, with many studies dedicated to this area.

KRAS, a member of the oncogenic RAS family, plays a crucial role in the cellular signalling pathways that control cell proliferation, differentiation, and survival [8]. Mutations in the *KRAS* gene, particularly in codons 12 and 13, lead to constitutive activation of the RAS-RAF-MEK-ERK pathway, thereby driving uncontrolled cell growth and carcinogenesis [9]. Despite extensive research, *KRAS* mutations remain a challenging target in cancer therapy, partly due to their inherent resistance to various therapeutic agents and their role in altering the tumour microenvironment [10]. NSCLC exhibits a wide molecular heterogeneity, driven by genomic instability, posing significant challenges for effective treatment [11]. Recent studies have highlighted significant prognostic factors in NSCLC related to recurrence risk following surgical resection. These factors, including tumour size, lymph node involvement, and genetic mutations, particularly in *EGFR* and *KRAS*, are essential for tailoring treatment strategies and improving patient outcomes [12]. Among these, mutations in the *KRAS* have been identified as a key driving factor in a substantial proportion of NSCLC patients.

This study utilises the TCGA dataset to examine the impact of *KRAS* mutations on the gene expression profile in NSCLC, and to explore related changes in the tumour microenvironment, particularly in terms of immune cell infiltration and response to therapy.

Material and methods

Data acquisition and analysis

Data of RNA sequencing and whole-exome sequencing for 978 NSCLC samples were obtained from The Cancer Genome Atlas (TCGA) database, which included comprehensive gene expression profiles and detailed mutation information for each sample. After quality control checks on the raw sequencing data, the RNA-Seq data were normalised using FPKM (fragments per kilobase of transcript per million mapped reads). The tumour mutational burden (TMB) of each patient in this cohort was recalculated using a maftools method [13].

Subsequently, each NSCLC sample was categorised based on its *KRAS* mutation status (mutant or wild type). Then differential expression analysis was performed using the Deseq2 package in R [14] to identify differentially expressed genes (DEGs) between *KRAS* mutant and wild-type groups. The screening threshold was $|\log2 \text{ fold change}| > 1$ and $p < 0.05$.

Gene set enrichment analysis (GSEA) [15] was carried out to identify the Kyoto Encyclopedia of Genes and Genomes (KEGG) in the *KRAS* mutant and wild-type groups. Enrichment analysis of DEGs was conducted using the clusterProfiler package in R [16] to identify the biological process (BP), cellular component (CC), and molecular function (MF) of the gene ontology (GO) and KEGG. $P < 0.05$ was considered as significant enrichment.

Immune dysfunction analysis

Single-sample gene set enrichment analysis (ssGSEA) was performed to quantify the infiltration of 28 immune cell types in each samples using the GSVA package in R [17]. Tumour immune dysfunction and exclusion (TIDE) scores were computed for each NSCLC sample to evaluate the dysfunction of tumour-infiltrating cytotoxic T lymphocytes and immunosuppressive factors in the tumour microenvironment. Higher TIDE scores indicate a greater likelihood of immune evasion.

In addition, SubMap analysis was conducted to contrast the responders of anti-PD-1 or anti-CTLA-4 therapy between *KRAS* mutant and wild-type groups. The Genomics of Drug Sensitivity in Cancer (GDSC) database [18] was used to predict the chemosensitivity and the half-maximal inhibitory concentration (IC50) of *KRAS* mutant and wild-type groups by pRRophetic package in R [19].

Patient cohort selection

Blood and tumour tissue samples were obtained from 10 *KRAS*-G12C, 10 *KRAS*-G12D, 10 *KRAS*-G12V, and 10 *KRAS* wild-type NSCLC patients from the Affiliated Cancer Hospital of Xinjiang Medical University between April 2022 and March 2023. The inclusion criterion was patients diagnosed with NSCLC based on histopathological analysis. Exclusion criteria included patients with a history of other primary malignancies within the last 5 years; patients who received any form of cancer therapy (chemotherapy, immunotherapy) within a month prior to sample collection; and patients with severe comorbid conditions (autoimmune diseases, chronic infections). This study was approved by the Ethics Committee of the Affiliated Cancer Hospital of Xinjiang Medical University (No. K2022040). Informed consent was obtained from each patient.

Western Blot

We extracted proteins from tissue samples using RIPA buffer. The protein concentration was then determined with a BCA reagent kit (Beyotime, Shanghai, China). Equal amounts of protein samples were mixed with SDS sample buffer, and the proteins were denatured by heating. Next, the denatured protein

samples were loaded onto an SDS-PAGE gel and separated using an electric current. After electrophoresis, the separated proteins were transferred onto a polyvinylidene fluoride membrane. The membrane was incubated with skimmed milk at room temperature for 2 hours. The membrane was then incubated with specific primary antibodies (Abcam, CA, UK) followed by corresponding secondary antibodies. Protein bands were detected with chemiluminescent substrate in a chemiluminescent imaging system. GAPDH was used as the internal reference protein, and the relative protein expression levels were calculated using ImageJ software (NIH, MD, USA).

Flow cytometry

After lysis of red blood cells, blood samples were stained with fluorescently labelled antibodies targeting surface markers: anti-human CD45-APC, CD8-FITC, PD-1-PC7, and CD152-ECD antibodies (BD Biosciences, CA, USA) for 30 min at 4°C. Samples were measured under a flow cytometry (BD Biosciences). Data analysis begins with gating on live cells based on SSC profiles. Subsequent gates are applied to identify specific cell populations: CD8+PD-1 and CD8+CTLA-4 cells. Data are processed using FlowJo software (Tree Star).

Statistical analysis

R software (v3.5.1) was used for bioinformatics analysis. Statistical analysis was performed with GraphPad Prism software (v9.3.0). Data are presented as the mean ± standard deviation (SD). Statistical significance was determined using *t*-test or ANOVA. $P < 0.05$ was considered significant.

Results

Mutational landscape and frequency of *KRAS* mutations

To identify the somatic mutations among the NSCLC patients in the TCGA database, a summary of the mutation information was visualised in Figure 1A. *KRAS* were mutated in 15% of samples. The correlation calculations shown some co-occurrence or exclusive mutations between *KRAS* mutations and some gene mutations (Fig. 1B). The transition-transversion (TiTv) plots showed the transition mutations; cytosine to thymine (C>T) was the highest (Fig. 1C). The mutated rate of *KRAS*-G12C, *KRAS*-G12V, and *KRAS*-G12D were found to be the most frequent (Fig. 1D), highlighting their predominant role in NSCLC pathogenesis.

Analysis of differentially gene expression

We identified a total of 1323 DEGs when comparing *KRAS* mutant to *KRAS* wild-type NSCLC samples (Fig. 2A, B), which including 532 up-regulated DEGs and 791 down-regulated DEGs. Enrichment analysis showed that these DEGs were significantly enriched in PI3K-Akt signalling pathway, cell adhesion molecules (CAMs), and metabolism of xenobiotics by cytochrome P450 (Fig. 2C). We found that KRAS and PI3K-Akt signalling pathways in GSEA were activated more in *KRAS* mutant than in *KRAS* wild-type samples (Fig. 2D).

Immune landscape alteration in *KRAS* mutant NSCLC

Furthermore, we depicted the immune microenvironment in *KRAS* mutant versus wild-type NSCLC. Through ssGSEA, we found a generally lower level of immune cell infiltration in *KRAS* mutant NSCLC compared to the wild-type type (Fig. 3A), including activated CD4 T cell, central memory CD4 T cell, effector memory CD4 T cell, memory B cell, CD56bright natural killer cell, natural killer T cell, and plasmacytoid dendritic cell. Our data showed a higher TIDE score, microsatellite instability (MSI) score, exclusion, and myeloid-derived suppressor cell (MDSC) in the *KRAS* mutant than in the *KRAS* wild-type group (Fig. 3B).

At the same time, patients with *KRAS* mutant were more sensitive to anti-PD-1 and anti-CTLA-4 therapy using SubMap analysis (Fig. 3C). The prediction of the sensibility of chemotherapeutics was conducted to evaluate the IC50 value (Fig. 3D). We found that the *KRAS* mutant showed lower IC50 for responding to BMS.509744, AS601245, JNK.9L, DMOG, BMS.536924, FH535, Embelin, Mitomycin.C, AZD6244, and BMS.754807.

Validation of the protein expression

Western blot analyses revealed that the expression levels of immune checkpoint proteins PD-L1 and CTLA-4 were significantly higher in *KRAS* mutant than *KRAS* wild-type NSCLC samples (Fig. 4A). Flow cytometry showed a higher presence of CD8+PD-1+ and CD8+CTLA-4+ T cells in *KRAS* mutant than *KRAS* wild-type NSCLC samples (Fig. 4B).

Discussion

KRAS mutations are the most common molecular alteration in patients with advanced NSCLC. Our findings from the TCGA database analysis have elucidated the significant role of *KRAS* mutations in the pathogenesis of NSCLC. The predominance of specific *KRAS* mutations, particularly *KRAS*-G12C, *KRAS*-G12D, and *KRAS*-G12V, highlights their significant role in NSCLC development. The mutation leading to the replacement of glycine by cysteine

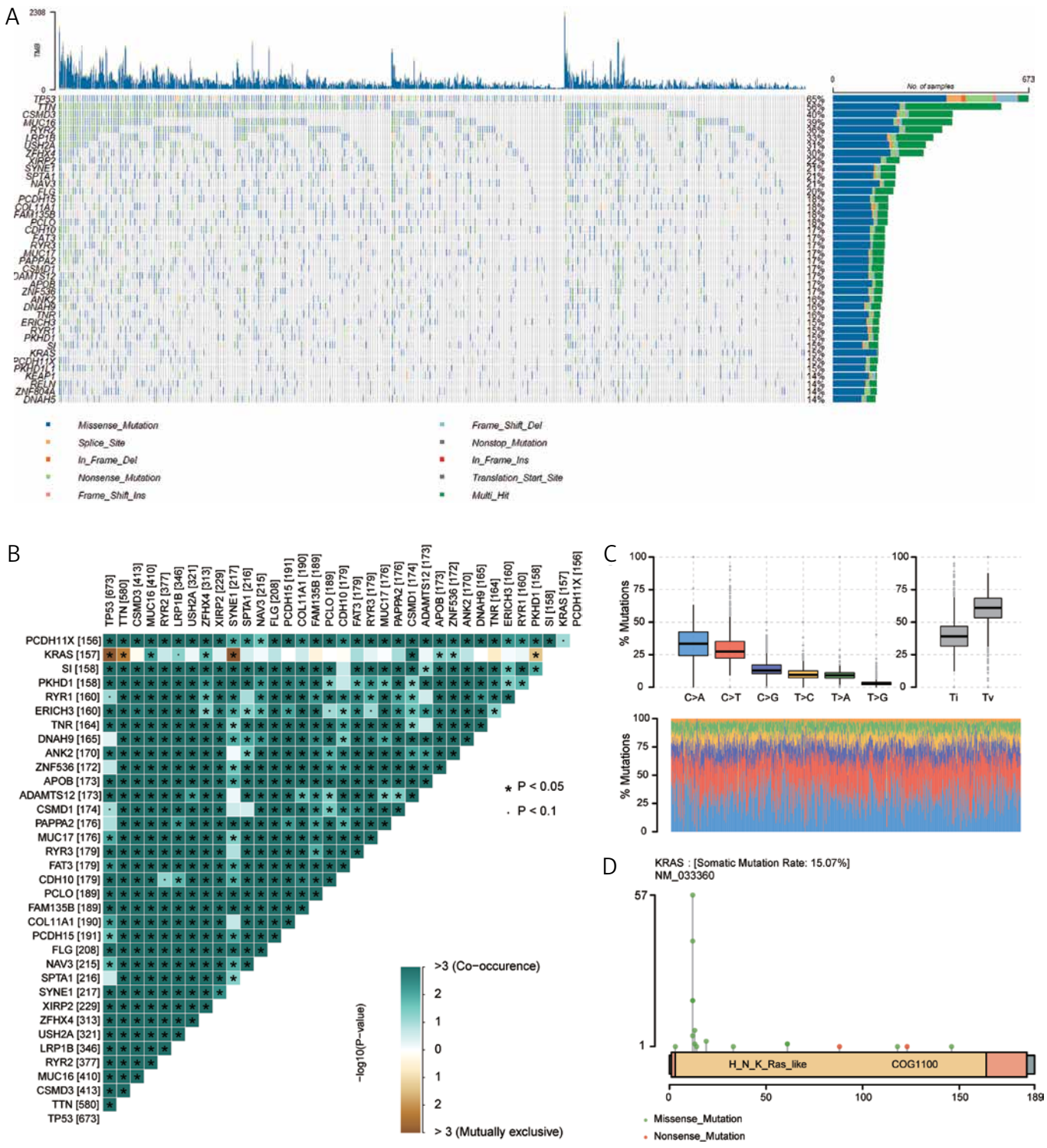

Fig. 1. Analysis of KRAS mutation profiles in NSCLC. A) Mutation summary plots of detailed information for the top 40 genes in each sample. TMB, tumour mutational burden. B) Correlation between the top 35 mutated genes. C) Fraction of transition-transversion (TiTv) conversions. D) Lollipop plot of mutation distribution and protein domains for KRAS

(*KRAS*-G12C) is the most common *KRAS* mutation in non-small cell lung cancer, accounting for 40% of all *KRAS* mutation cases, followed by the replacement of glycine by valine (*KRAS*-G12V, 21%) and glycine by aspartic acid (*KRAS*-G12D, 17%) [20]. These mutations may confer distinct biological behaviours and therapeutic vulnerabilities, which could be pivotal in guiding personalised treatment strategies.

Our identification of DEGs in *KRAS* mutant versus wild-type NSCLC provides insights into the molecular mechanisms underlying *KRAS*-driven oncogenesis. The significant enrichment of these DEGs in pathways like PI3K-Akt signalling, cell adhesion molecules (CAMs), and metabolism of xenobiotics by cytochrome P450 points to a complex interplay of cellular processes influenced by *KRAS* mutations.

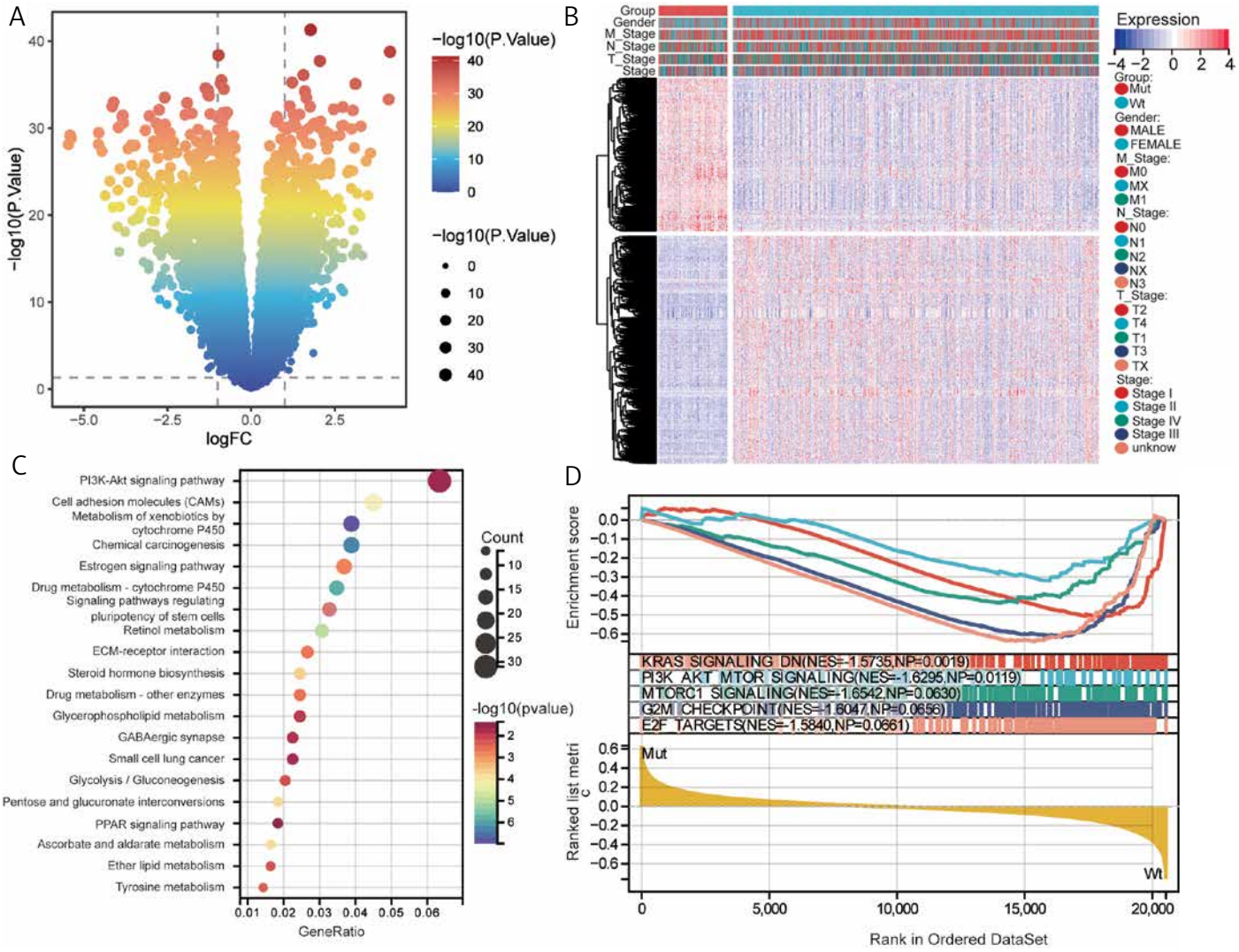

Fig. 2. Differentially expressed genes between KRAS mutant and wild-type NSCLC samples. A) Volcano plot of differentially expressed genes. B) Expression heatmap of differentially expressed genes in KRAS mutant and wild-type samples. C) KEGG pathway enriched by differentially expressed genes. D) GSEA results showed KEGG pathways in KRAS mutant and wild-type samples. NES, normalised enrichment score

When *KRAS* is mutated, RAS is locked in a GTP-bound active form, which constitutively activates downstream signalling pathways such as the PI3K-AKT-mTOR pathway, leading to malignant phenotypes [21]. In fact, hydrophobic G12C and G12V preferentially activate the RAL pathway, while hydrophilic G12D primarily functions through PI3K-AKT signalling [22]. CAMs promote tumour metastasis by binding with LFA-1 in NSCLC cells and they facilitate tumour tissue aggregation, impairing the recirculation of CD8+ T cells [4]. Flavonoid compounds with higher binding affinity to *KRAS*-G12D mutated protein affect cytochrome p450, regulating the cell cycle [23]. Notably, the activation of the PI3K-Akt pathway in *KRAS* mutant samples, as indicated by GSEA, might contribute to enhanced cell survival, proliferation, and resistance to apoptosis, which are hallmarks of cancer progression. Cell lines with *KRAS*-G12C and *KRAS*-G12V mutations exhibit higher RAL activity and lower AKT phosphorylation, whereas cell lines with G12D mutations show higher PI3K-AKT activity compared to other mutations and the wild type [24]. *KRAS*-G12C and -G12V mutations tend to activate the RAL signalling pathway and show lower PI3K-Akt activity, while *KRAS*-G12D mutations increase PI3K-Akt signalling [25]. The divergence between our findings and the cited work probably arises from differences in the types of mutations analysed. Our GSEA encompassed a broader range of *KRAS* mutations, potentially diluting the effects of individual mutations [26]. Further studies focusing on mutation-specific signalling would be needed to fully understand the role of the PI3K-Akt pathway in different *KRAS* mutation contexts.

Our study revealed a generally lower level of immune cell infiltration in *KRAS* mutant NSCLC compared to wild type. This reduced infiltration, particularly of cells like activated CD4 T cells, memory B cells, and natural killer cells, could contribute to an immunosuppressive tumour environment, facili-

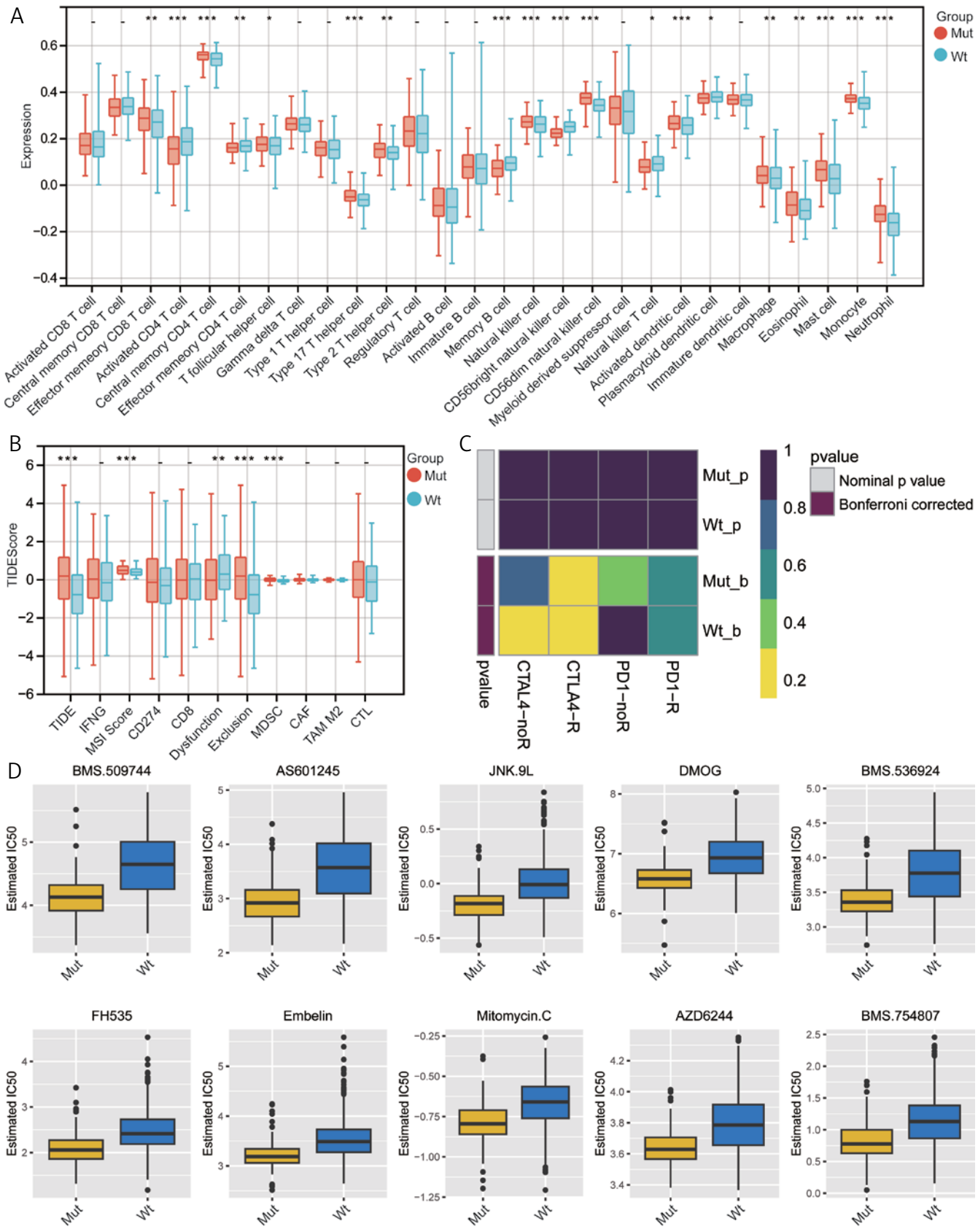

Fig. 3. Immune and therapy sensitivity in KRAS mutant NSCLC. A) The infiltration level of immune cells in KRAS mutant and KRAS wild-type NSCLC. B) Dysfunction/exclusion scores by TIDE database in KRAS mutant and KRAS wild NSCLC. TIDE, tumour immune dysfunction and exclusion; MSI, microsatellite instability; MDSC, myeloid-derived suppressor cell; CAF, cancer-associated fibroblasts; TAM, tumour-associated macrophages; CTL, cytotoxic T lymphocyte. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. C) Submap analysis for KRAS mutant and KRAS wild-type groups. (D) Prediction of chemotherapeutic sensibility for KRAS mutant and KRAS wild-type groups

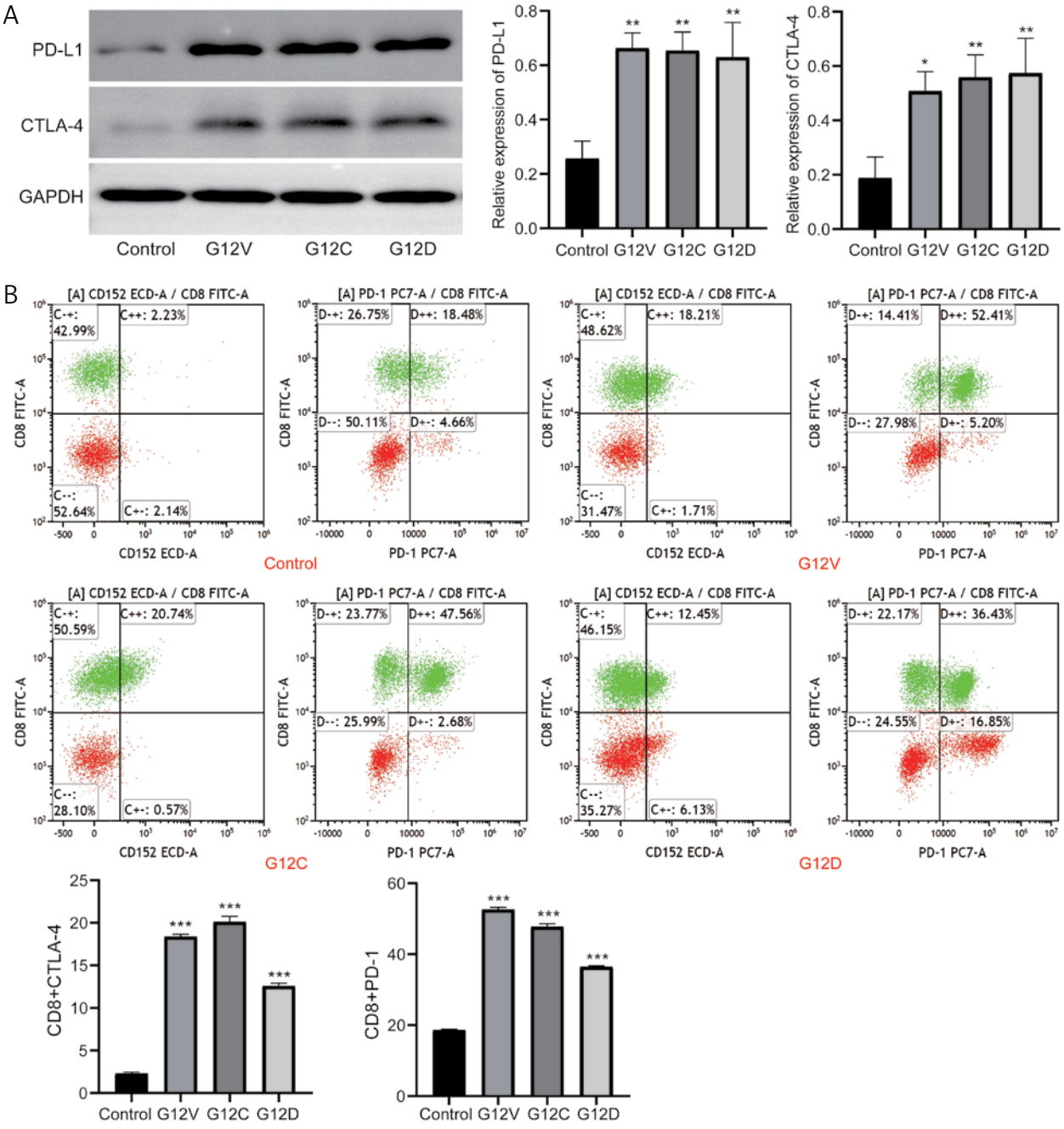

Fig. 4. Detection of expression of PD-1/PD-L1 and CTLA-4. A) Protein expression of protein expression of PD-L1 and CTLA-4 in KRAS mutant than KRAS wild-type NSCLC samples detected by Western blot. B) The proportion of CD8+PD-1+T cells and CD8+CTLA-4+T cells detected by flow cytometry. ***$p < 0.001$

tating tumour immune evasion [27]. Clinical efficacy has been observed in *KRAS*-targeted therapies; however, patients still exhibit primary or acquired resistance to monotherapy [28]. The combination of *KRAS* inhibitors and ICIs may enhance PD-L1 expression and render cancer cells more sensitive to immune checkpoint inhibition [29].

Additionally, the higher TIDE score, MSI score, and the presence of exclusion and MDSCs in *KRAS* mutant samples further support the notion of an immune-resistant tumour microenvironment. Consistent with others, *KRAS* mutations are associated with high levels of CD8+PD-1+T cells and CD8+CTLA-4+T cells and a high TIDE score [30].

The more sensitivity of *KRAS* mutant NSCLC to anti-PD-1/PD-L1 and anti-CTLA-4 therapies is a critical finding. *KRAS*-G12C mutated non-small cell lung cancer exhibits higher levels of PD-L1 compared to non-G12C mutations [31]. Treatment outcomes for patients with *EGFR*-mutant NSCLC, especially those

with brain metastases, have been shown to improve with the combination of EGFR-TKI and locoregional therapies. These findings underscore the importance of integrating multimodal approaches to manage advanced NSCLC effectively [32]. However, the clinical outcomes for *KRAS* mutated patients undergoing immunotherapy remain unclear. It suggests that patients with *KRAS* mutations may require combination therapies or alternative treatment strategies. Our findings from Western blot analyses and flow cytometry further corroborate the hypothesis of an immune-suppressed state in *KRAS* mutant NSCLC. The elevated expression levels of PD-L1 and CTLA-4 in *KRAS* mutant samples could lead to insufficient immune activation generated by targeting CTLA-4 or PD-1 to control tumour progression [33]. Similarly, the increased presence of CD8+PD-1+ and CD8+CTLA-4+ T cells in *KRAS* mutant NSCLC might reflect a tumour microenvironment that is adept at evading immune-mediated destruction [34, 35].

The increased sensitivity of *KRAS* mutant NSCLC to several chemotherapeutics, as reflected in the lower IC50 values for drugs like BMS-509744 (ITK inhibitor), AS601245 (JNK inhibitor), AZD6244 (MEK inhibitor), and others, poses a significant management of these patients. ITK inhibitors suppress downstream activation of Th2 cells, providing a potential compensatory platform for the activation of Th1 and CD8 T cells [36]. *KRAS* inhibition reduces activation levels of ERK, JNK, PI3K, modulating cell proliferation, autophagy, adipogenesis, and lipid accumulation [37]. The use of MEK inhibitors offers some limited benefits in treating patients with *KRAS*-mutated NSCLC [38]. *KRAS* resistance can also be mediated through the activation of bypass signalling pathways, enabling the MAPK or PI3K pathways to continue signalling even when *KRAS* is fully inhibited [39]. This resistance might be attributable to the altered signalling pathways and the immunosuppressive tumour microenvironment driven by *KRAS* mutations.

There are some limitations in this study. Our research is based on retrospective analysis of the TCGA database, leading to inherent limitations such as selection bias and the lack of longitudinal data, also potentially limiting the generalisability of our findings. Furthermore, our study focuses primarily on genetic and transcriptomic data, and does not incorporate proteomic or metabolomic analyses, which might provide additional insights. The study lacks direct correlation with clinical outcomes, such as patient survival or detailed treatment response. The translational potential of our findings, therefore, remains to be validated in clinical settings. Prospective studies and clinical trials are needed to assess the applicability of our findings in therapeutic decision-making for NSCLC patients with *KRAS* mutations.

Conclusions

Our study provides a comprehensive understanding of the impact of *KRAS* mutations on the molecular and immunological landscape of NSCLC. The findings underscore the need for developing tailored therapeutic strategies that consider the unique characteristics of *KRAS* mutant NSCLC, including its resistance to conventional chemotherapies and immunotherapies. Further research is warranted to develop effective strategies to overcome the challenges posed by *KRAS* mutations in lung cancer therapy.

Disclosures

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4. Conflicts of interest: None.

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