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Analysis

Integrated multi-omics characterization of SMAD4 mutant colorectal cancer

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Abstract
Colorectal cancer is one of the most common cancers around the world, which is a severe threat to people's health. SMAD4 belongs to the dwarfin/SMAD family, which plays a crucial role in TGF-β and BMP signal pathways. As the molecular characterization of colon cancer patients following SMAD4 mutations remains unclear, we integrated multi-omics data of SMAD4 mutant patients to reveal the profile of molecular characterization of SMAD4 mutation. A missense mutation is the most common mutant type of SMAD4. Patients with SMAD4 mutation had worse survival. Tumor tissues from patients carrying the SMAD4 mutation showed a reduction in various immune cells, such as CD4 + memory T cells and memory B cells. Many differential genes were identified compared to the SMAD4 mutation-free group and could be significantly enriched for tumor- and immune-related signaling pathways. In addition, the mutant group had different drug sensitivities than the non-mutant group.

Keywords Colorectal cancer · SMAD4 · Multi-omics data · Single cell RNA sequence

1 Introduction

SMAD4 gene, also known as the DPC4 gene, is located on the long arm of chromosome 18 and encodes a protein called SMAD4. It is a tumor suppressor gene that helps prevent cancer development by regulating cell growth and differentiation, and it is often mutated in various types of cancer [1]. SMAD4 is a transcription factor that plays a critical role in the transforming growth factor-beta (TGF-β) signaling pathway and is involved in multiple cellular processes, including cell proliferation, differentiation, apoptosis, and migration [2]. When TGF-β ligands bind to their receptors, SMAD4 is activated and translocated into the nucleus, where it acts as a transcription factor, regulating the expression of target genes [3]. Loss-of-function mutations in the SMAD4 gene have been reported in several types of cancer, including pancreatic, colorectal, gastric, and ovarian cancers [4–8]. These mutations decrease SMAD4 activity, resulting in dysregulated TGF-β signaling and aberrant cell growth and proliferation.

Danyi Zhao and Yanjun Qu contributed equally to this work. Na Gao and Tao Wu were corresponding authors for the study.

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SMAD4 gene mutations are prevalent in tumors. Of these, approximately 10–30% of colorectal cancer cases are found to carry mutations in the SMAD4 gene [9]. Similarly, up to 55% of pancreatic cancer patients harbor mutations in this gene [10]. This mutation makes the disease more aggressive and is associated with a poorer prognosis [11, 12]. However, SMAD4 mutations may also have therapeutic implications. Preclinical studies have shown that re-expression of functional SMAD4 in SMAD4-deficient cancer cells can lead to growth suppression and sensitization to chemotherapy [13]. Therefore, strategies aimed at restoring SMAD4 function may have potential therapeutic value in certain types of cancer.

Research on the SMAD4 gene has also revealed its involvement in other diseases and conditions beyond cancer. For example, mutations in the SMAD4 gene can cause a rare genetic disorder known as juvenile polyposis syndrome (JPS) [14, 15]. JPS is characterized by multiple polyps in the gastrointestinal tract, particularly in the colon and rectum. These polyps have the potential to become cancerous if left untreated [16]. Along with gastrointestinal polyps, individuals with JPS may also have other features, such as pigmented spots on the lips and hands, as well as a predisposition to certain types of tumors. Studies have suggested that the SMAD4 gene may be involved in the pathogenesis of cardiovascular disease. Research has shown that SMAD4 plays a role in the development and function of blood vessels, and disruptions in SMAD4 signaling may contribute to abnormal blood vessel growth and cardiovascular disorders [17].

Understanding the function and regulation of the SMAD4 gene has important implications for both research and clinical practice. It provides insights into the underlying molecular mechanisms of cancer development and progression, offering potential targets for therapeutic interventions. Furthermore, identifying SMAD4 mutations in specific cancers can have diagnostic and prognostic implications, helping to guide treatment decisions and predict patient outcomes. Thus, the main objective of this study was to explore the altered transcriptome, immunity, and drug sensitivity of patients with SMAD4 mutant colorectal cancer through multi-omics data.

2 Materials and methods

2.1 Data acquirement and analysis

Somatic mutation data of colorectal cancer were downloaded from the TCGA. The transcriptome RNA sequence data were downloaded from UCSC Xena. The cBioportal (https://www.cbioportal.org/) was applied to plot the mutant lollipop. cBioPortal plotted the survival cure between the SMAD4 mutant and non-mutant groups. The tumor mutation burden was calculated using the somatic mutant file of colorectal cancer. The "maftools" was used to analyze the mutant waterfall plot.

2.2 Single cell and immune microenvironment analysis

Single-cell analysis of SMAD4 mutant and non-mutant samples (GSE132465) was downloaded by Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). The t-SNE algorithm was applied to single-cell cluster analysis. Serut analyzed single-cell analysis. The annotation of single-cell RNA data was conducted by "SingleR". Microenvironment infiltration of immune cells was evaluated by the cell algorithm through the R package of IOBR.

2.3 Correlation of immune related gene

All immune-related genes were downloaded from IMMPORT (https://www.immport.org/home).

Patients of colorectal cancer were divided into two groups, with mutant and non-mutant. Immune-related genes between the two groups were analyzed using the Wilcox test.

2.4 Differential genes between mutant- and non-mutant groups

The differential expressed R packages of "limma" enumerated genes. Enrichment analysis of differential genes is mainly done by "clusterProfiler". The enrichment pathway gene sets were downloaded from the MsigDB (https://www.gsea-msigdb.org/gsea/msigdb). Visualization is principally done through ggplot2.

2.5 Drug sensitivity analysis between mutant- and non-mutant groups

The oncoPredict was applied to calculate the relative drug sensitivity. The drug sensitivity value was tumor-related drugs or molecular compounds based on Genomics of Drug Sensitivity in Cancer.

2.6 Statistical Analysis

All the data was processed by R 4.3.0. Wilcox, unpaired and paired student t-tests for the continuous variable and chi-square test for dichotomous variables were applied to determine the statistical significance of differences between mutant- and non-mutant groups. Unless otherwise noted, p-value ≤ 0.05 was used to determine statistical significance for all tests.

3 Results

3.1 Clinical difference between mutant and non-mutant group

In the clinical factor analysis, 437 patients were included in the TCGA cohort, of which 49 colon cancer patients carried SMAD4 gene mutations. The mean age of the mutated and non-mutated groups was 68.9 ± 12.6 and 66.4 ± 13.1, respectively (p = 0.207). There was no significant difference between the two groups regarding the history of colon polyps (p = 0.499). There may be some difference in mismatch repair protein between the two groups (p = 0.029). In addition, the two groups had a significant difference in BMI. Other clinical factors (TNM stage, number of positive lymph nodes, etc.) did not show any difference between the two groups. Table 1 shows the detailed information.

3.2 Mutation profile

Using the cBioPortal, we plotted a lollipop map of mutations in the SMAD4 gene (Fig. 1A). Its mutation rate was about 10.99%, and the primary type of mutation was missense mutation. To investigate whether the SMAD4 mutation would lead to differences in patients' prognosis, we plotted the survival curves of patients with or without the mutation by the cBioPortal, and the results showed that the prognosis of patients harboring the SMAD4 mutation became worse. It implies that the prognosis of patients without SMAD4 mutation is significantly better than that of patients carrying SMAD4 mutation (Fig. 1B). Further, we explored the relationship between tumor mutation load and SMAD4 gene mutation and found that TMB was elevated in patients with SMAD4 mutation compared with the unmutated group (Fig. 1C).

To clarify the specific characteristics of SMAD4 mutations, we drew a waterfall plot of patients who carried mutations in the SMAD4 gene. In the SMAD4 mutation group, the top 6 mutated genes were APC, TNN, KRAS, FAT4, MUC16 and TP53 (Fig. 1D). Figure 1E demonstrates the significantly different genes between the mutant and non-mutant groups. The mutation profile differed from that of the patients who did not carry the SMAD4 gene.

3.3 Single cell analysis and immune infiltration analysis

To clarify whether there is a correlation between SMAD4 mutation and tumor microenvironment, we downloaded 4 patients with SMAD4 mutation from GEO and 4 patients without mutation for control analysis. Figure 2A (Left) demonstrates the overall clustering distribution. Figure 2A (Middle) reflects the distribution of various immune cells. Figure 2A (Right) presents the distribution of SMAD4 mutations. Figure 2B shows the proportion of different types of cells in the 8 patients, with some variability between the mutated and non-mutated groups. To facilitate quantitative analysis of the differences between the two groups, we performed immune infiltration analysis on bulk RNA sequencing data from TCGA, and the results showed significant differences in a variety of immune cells, such as CD4 + memory T cells, memory B cells, etc. (Fig. 2C).

3.4 Immune-related genes

Immune-related genes were extracted from bulk RNA sequencing of TCGA datasets, and the Wilcox test was performed to detect any differences between mutant and non-mutant groups. Figure 3 shows the part of differentially expressed immune-related genes between the two groups.

Table 1 The clinical characteristics of patients

	Mutant N = 49	Non-mutant N = 388	P-value
Age	68.9 (12.6)	66.4 (13.1)	0.207
History of colon polyps			0.499
NO	25 (51.0%)	232 (59.8%)	
YES	3 (6.12%)	55 (14.2%)	
Not reported	21 (42.9%)	101 (26.0%)	
Loss expression of mismatch repair proteins by IHC			0.029
NO	25 (51.0%)	232 (59.8%)	
YES	3 (6.12%)	55 (14.2%)	
Not reported	21 (42.9%)	101 (26.0%)	
Lymphatic invasion			0.369
NO	25 (51.0%)	209 (53.9%)	
YES	16 (32.7%)	141 (36.3%)	
Not reported	8 (16.3%)	38 (9.79%)	
Non nodal tumor deposits			0.653
NO	20 (40.8%)	174 (44.8%)	
YES	3 (6.12%)	36 (9.28%)	
Not reported	26 (53.1%)	178 (45.9%)	
Number of lymph nodes positive by HE	2.42 (4.04)	2.06 (4.54)	0.586
M			0.976
M0	34 (69.4%)	275 (70.9%)	
M1	8 (16.3%)	57 (14.7%)	
MX	6 (12.2%)	48 (12.4%)	
Not reported	1 (2.04%)	8 (2.06%)	
N			0.982
N0	28 (57.1%)	226 (58.2%)	
N1	12 (24.5%)	89 (22.9%)	
N2	9 (18.4%)	71 (18.3%)	
Not reported	0 (0.00%)	2 (0.52%)	
T			0.86
Tis	0 (0.00%)	1 (0.26%)	
T1	1 (2.04%)	9 (2.32%)	
T2	6 (12.2%)	68 (17.5%)	
T3	35 (71.4%)	260 (67.0%)	
T4	7 (14.3%)	48 (12.4%)	
Not reported	0 (0.00%)	2 (0.52%)	
CEA	38.1 (103)	39.3 (211)	0.961
Venous invasion			0.296
NO	27 (55.1%)	252 (64.9%)	
YES	12 (24.5%)	85 (21.9%)	
Not reported	10 (20.4%)	51 (13.1%)	
Gender			0.123
Female	30 (61.2%)	180 (46.4%)	
Male	19 (38.8%)	206 (53.1%)	
Not reported	0 (0.00%)	2 (0.52%)	
Stage			0.966
I	7 (14.3%)	64 (16.5%)	
II	21 (42.9%)	146 (37.6%)	
III	12 (24.5%)	109 (28.1%)	
IV	8 (16.3%)	57 (14.7%)	

Table 1 (continued)

	Mutant N = 49	Non-mutant N = 388	P-value
Not reported	1 (2.04%)	12 (3.10%)	
BMI	27.0 (6.11)	31.0 (24.4)	0.049
Longest dimension	1.25 (0.45)	1.25 (0.56)	1

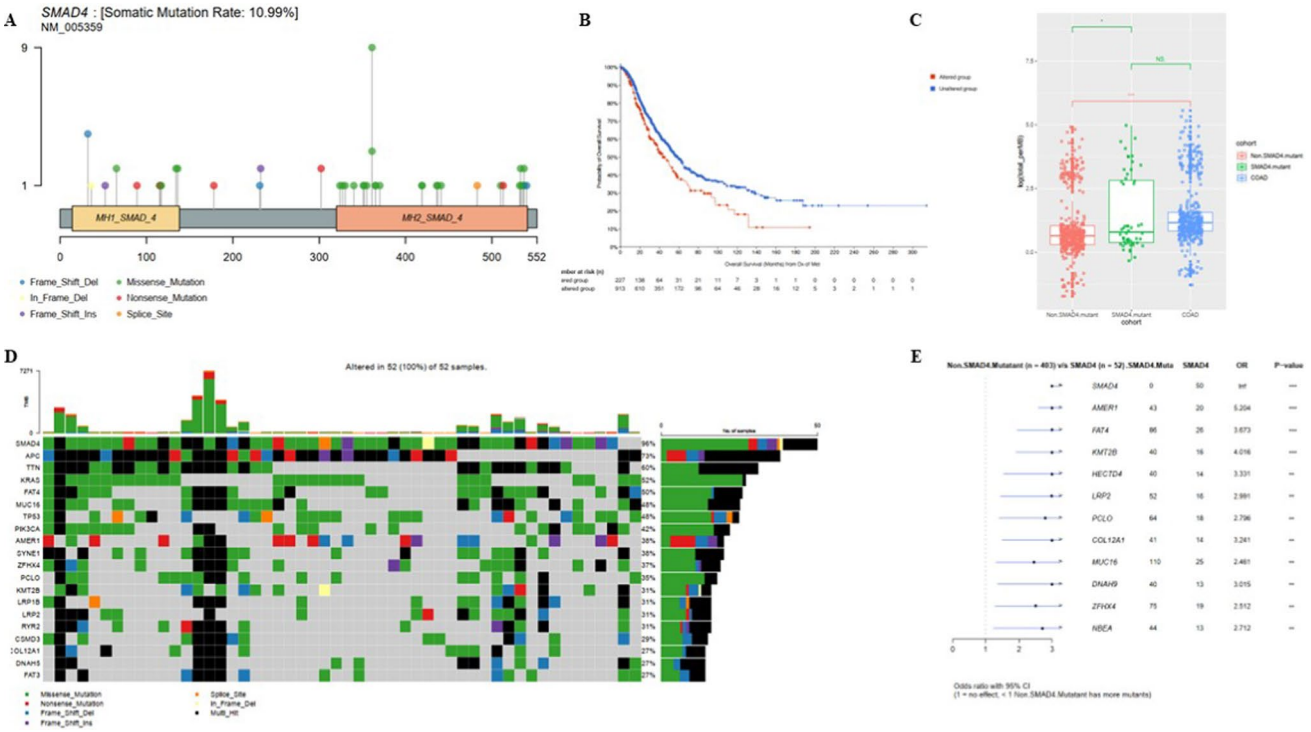


Fig. 1 The SMAD4 mutation profile in colorectal cancer. **A** The lollipop map of mutations in the SMAD4 gene. **B** Comparison of survival rate between the mutant and non-mutant groups of SMAD4. **C** The relationship between TMB and SMAD4 gene mutation. **D** The top 6 mutated genes in the SMAD4 mutation group. **E** The significantly different genes between the mutant and non-mutant groups

3.5 Differentially expressed genes

Based on the presence or absence of SMAD4 mutations, we divided the patients into two groups and performed differential gene expression analysis. We identified 36 upregulated genes and 34 down-regulated genes in the differential analysis. Figure 4A shows the volcano plot of the differential expressed genes. Figure 4B shows the top 10 genes with the most significant differences (5 upregulated and 5 downregulated). We performed GSEA enrichment analysis for the differential genes and identified some critical signaling pathways (Fig. 4D, E).

3.6 Drug sensitivity analysis

Drug sensitivity analysis showed that multiple monoclonal antibodies significantly differed between the two groups (Fig. 5).

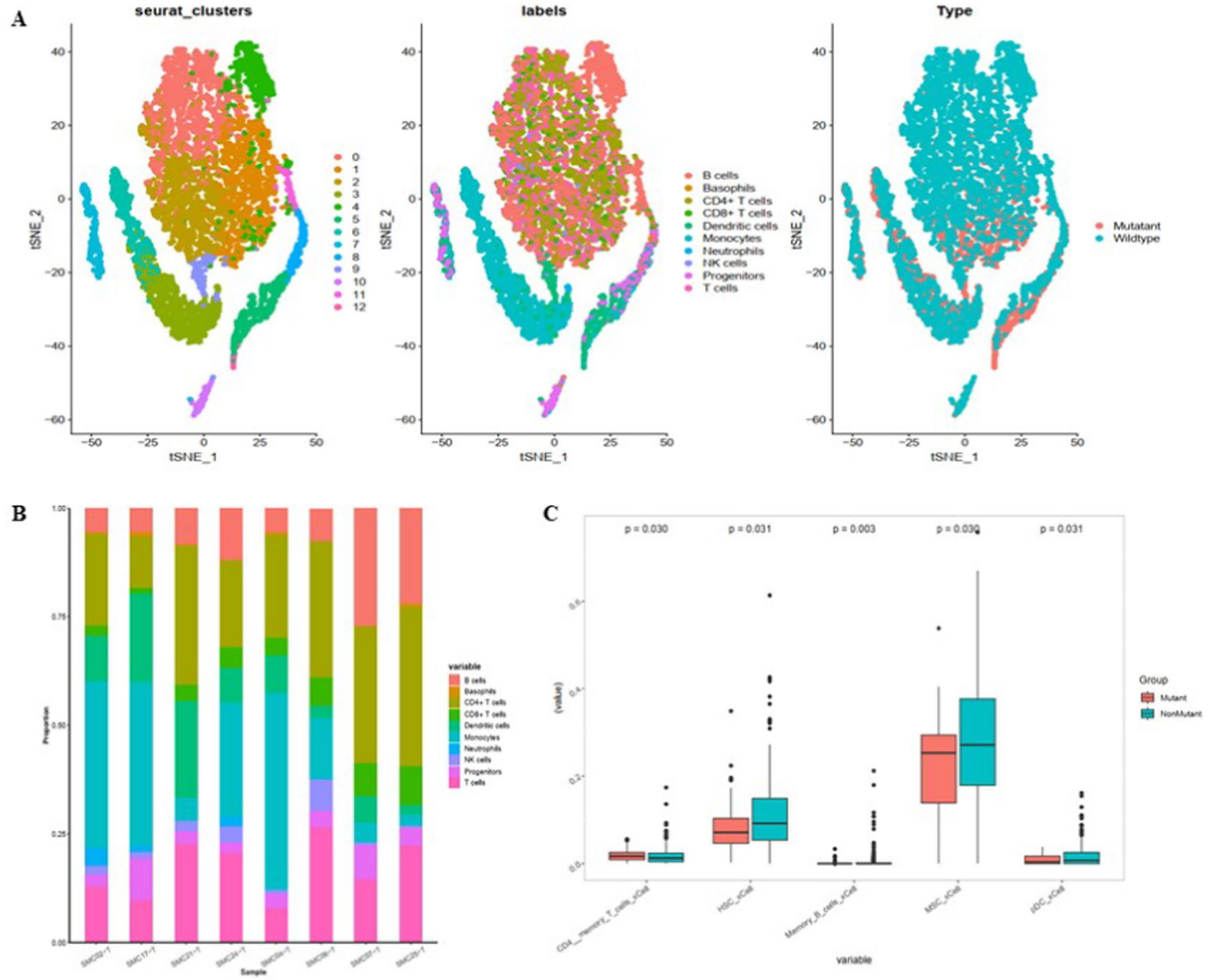


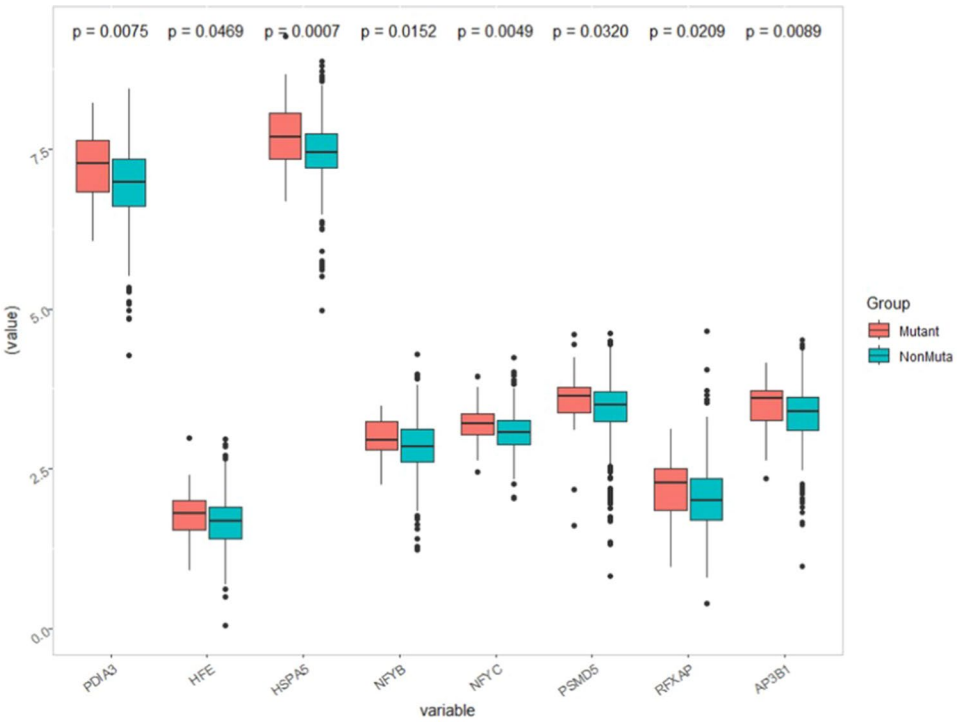
Fig. 2 The Single cell analysis and immune infiltration analysis. **A** The overall clustering distribution (left), the distribution of various immune cells (middle) and the distribution of SMAD4 mutations (right). **B** The proportion of different types of cells in the 8 patients, with some variability between the mutated and non-mutated groups. **C** The immune infiltration analysis based on TCGA data

4 Discussion

Cancer is a complex disease that involves abnormal cell growth and proliferation. Many genes and signaling pathways contribute to the development and progression of cancer. SMAD4 is a tumor suppressor gene critical to the TGF-β signaling pathway, which controls cell growth, differentiation, and apoptosis [18].

One of the first indications of SMAD4's involvement in cancer was observed in colorectal cancer [18]. Studies have shown that SMAD4 is frequently mutated or deleted in colorectal tumors. Loss of SMAD4 function in colorectal cancer leads to dysregulation of the TGF-β signaling pathway, promoting tumor growth and metastasis [19]. Moreover, SMAD4 inactivation is associated with poor prognosis and resistance to specific chemotherapeutic agents in colorectal cancer patients [20].

In pancreatic cancer, SMAD4 alterations are even more prevalent, with nearly 55–60% of cases showing SMAD4 gene mutations or deletions. Loss of SMAD4 function is associated with aggressive tumor behavior, increased invasiveness, and resistance to treatment. SMAD4 acts as a pancreatic tumor suppressor by regulating cell cycle control, cell proliferation, and apoptosis. Therefore, abnormalities in SMAD4 contribute to the development of pancreatic cancer and its poor prognosis [21].



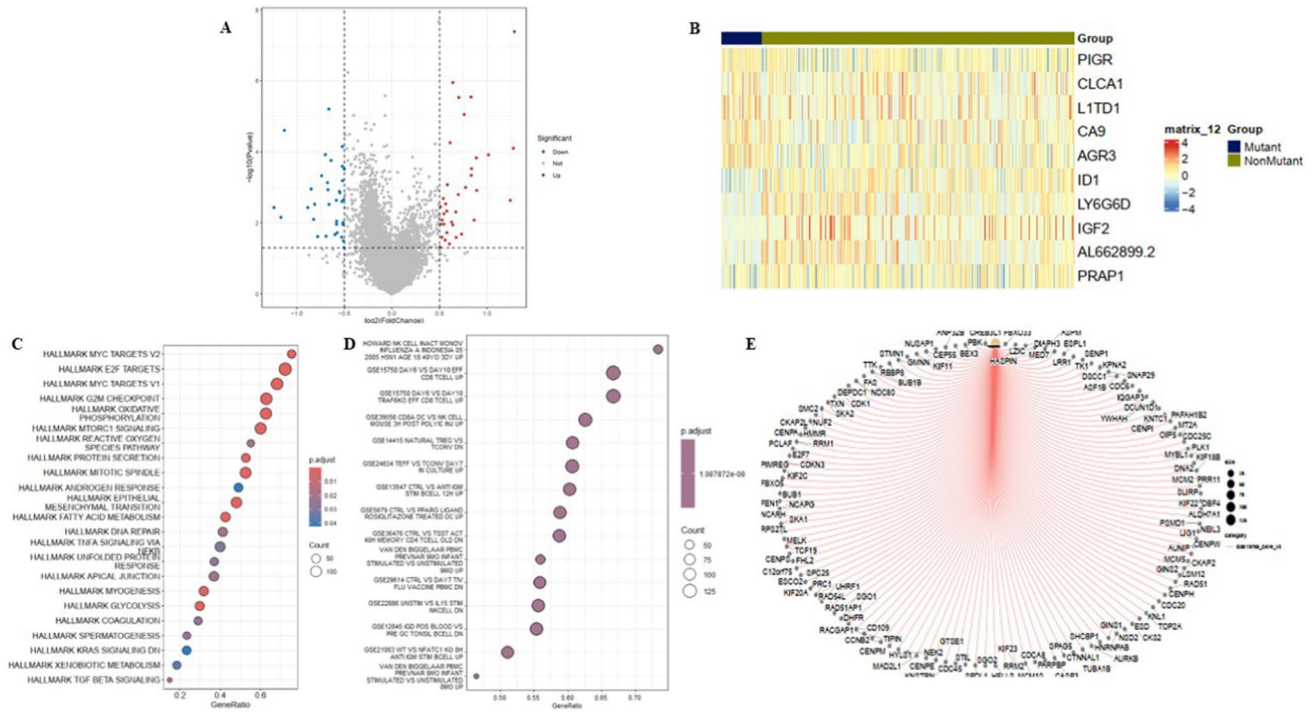


Fig. 4 The differentially expressed genes between the mutant and non-mutant groups. **A** The volcano plot of the differential expressed genes. **B** The top 10 genes with the most significant differences (5 upregulated and 5 downregulated). **C–E** The GSEA enrichment analysis and identification of some critical signaling pathways

Apart from colorectal and pancreatic cancers, dysregulation of SMAD4 has also been observed in other malignancies. SMAD4 gene alterations have been documented in gastric cancer, lung cancer, ovarian cancer, and others. In these tumors, SMAD4 inactivation has been linked to tumor progression, metastasis, and decreased patient survival. These findings highlight the universal importance of the SMAD4 gene in various types of cancer. Given the critical role of SMAD4

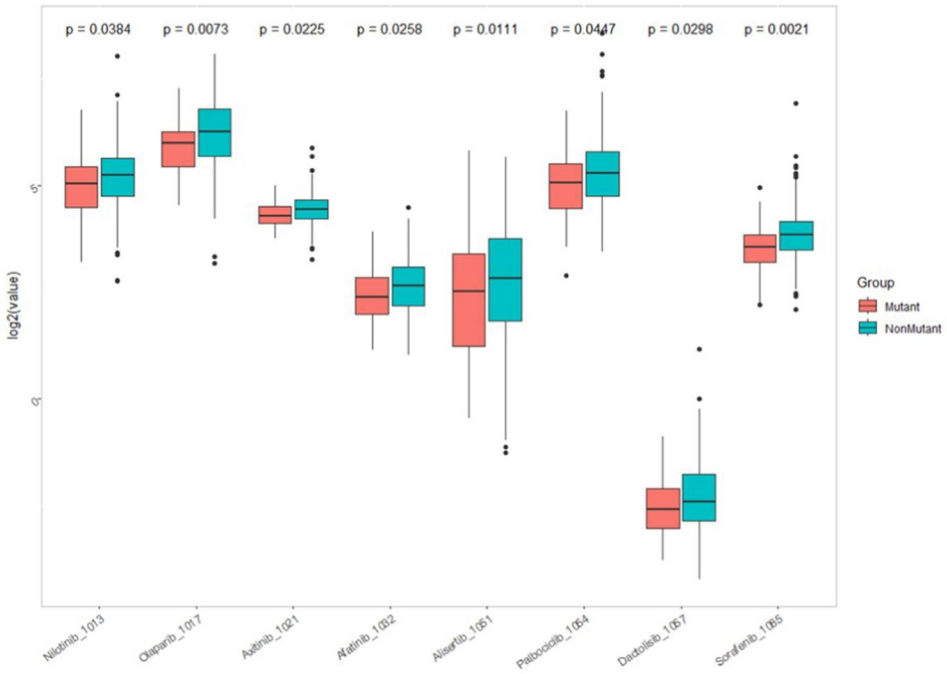


Fig. 5 Drug sensitivity analysis in mutant group and non-mutant group

in tumor suppression, targeting SMAD4 or the TGF-β signaling pathway holds promise as a therapeutic strategy [22]. Several approaches are being explored, including the restoration of SMAD4 function, inhibition of TGF-β signaling, and combination therapies. Gene therapies, such as gene replacement or targeted SMAD4 reactivation, are being investigated to restore the normal function of SMAD4 in cancer cells. Small molecule inhibitors and monoclonal antibodies targeting TGF-β receptors or downstream effectors are also being developed to block aberrant TGF-β signaling in tumors [23].

As the critical role of SMAD4, we comprehensively explored the molecular characteristics. In colorectal cancer, we found that patients with mutations in the SMAD4 gene had a worse prognosis than those without mutations. It is suggested that SMAD4 mutation results in the loss of cancer inhibitory effect. Tumor mutation load was elevated in patients in the SMAD4 mutation group compared with the unmutated group, implying that patients carrying SMAD4 mutations may be more likely to benefit from immunosuppressive therapy [24]. SMAD4 mutation status may serve as a predictor of sensitivity to immunosuppressive agents in patients with colorectal cancer.

The mutation profile of patients in the SMAD4 mutation group was significantly different from that of patients without SMAD4 mutations, and mutations in the SMAD4 gene may affect the mutation status of other genes [25]. Future studies are necessary to delve deeper into the role of SMAD4 mutations in somatic mutations.

Using single-cell sequencing data and tumor microenvironment immune infiltration analysis, we found that the tumor microenvironment of the patients changed significantly after the SMAD4 gene mutation. There was a significant difference in immune gene expression between the two groups. It is suggested that SMAD4 gene mutation can dramatically affect the immune status of the patients [26]. The CD4 + memory T cells were elevated, and the number of plasma-like dendritic cells was reduced [19]. At the same time, the differential gene enrichment analysis of the two groups suggested that multiple immune-related signaling pathways were altered. Multiple immune-related genes were also limited, and these changes indicated that SMAD4 gene mutations significantly affected the immune status of colon cancer patients [27].

We deeply analyzed the relationship between SMAD4 gene mutation status and drug sensitivity. We found that multiple monoclonal antibody drug sensitivities were altered between the two groups. This has implications for the treatment regimen of patients carrying mutations and is expected to lead to individualized medication for patients. We hope that these studies will be of assistance to clinicians.

However, it is undeniable that our study could be better. First, although we collected sequencing data from TCGA and GEO databases, the amount of these data still needs to be more significant for extensive data validation. There may be issues with sequencing data selection bias and poor representation of patient cohorts. Second, this study was only supported by bioinformatics analysis, and experimental support is needed further to clarify the mechanism of SMAD4 mutations in colorectal cancer. In the follow-up study, we will collect colorectal cancer tissue specimens from clinical

patients and verify the conclusions obtained in this study by HE staining, PCR, Western Blot and immunohistochemical staining. If sufficient funds are available, we will consider constructing SMAD4 mutated colorectal cancer cells and mouse models to validate the molecular mechanism further. We will construct different mutants in cancer cell lines to explore the role of SMAD4 mutants in colorectal cancer. In addition, mice with SMAD4 mutations will be bred for further study.

5 Conclusion

We analyzed the molecular characteristics between the mutated and non-mutated groups of the SMAD4 gene through multi-omics data. Colon cancer patients with SMAD4 gene mutations were found to have different immune infiltration than non-mutated patients. In the SMAD4 mutation group, the tumor mutation load was significantly more than in the non-mutated group. Multiple monoclonal antibody drug sensitivities were very different compared to the non-mutated group. SMAD4 gene mutation status may serve as a predictive marker for immunotherapy.

Author contributions WT and GN designed the experiments. ZD, QY and GN performed the experiments and contributed reagents/materials/analysis tools. ZD and QY wrote the paper. All authors have read and approved the manuscript.

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Data availability All datasets used and analyzed during the current study are available from the corresponding author upon reasonable request. Data is provided within the supplementary information files.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication All authors have read this manuscript and consented for publication.

Competing interests The authors declare no potential competing interests.

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