

# Comprehensive Analysis of the Expression and Prognostic Significance of NPAS1 in Patients with Colorectal Adenocarcinoma Based on Bioinformatics

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**Background:** Neuronal PAS domain protein 1 (NPAS1) is a protein-coding gene expressed mainly in the central nervous system and plays a key role in nervous system development. The expression and prognostic value of NPAS1 in colorectal adenocarcinoma (COAD) are unknown.

**Methods:** The expression and clinicopathological data of COAD from Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) were analyzed. NPAS1 gene expression in colon cancer tissues was validated by Western blotting and immunohistochemical staining. Function and immune infiltration were established using Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Set Enrichment Analysis (GSEA), and single sample Gene Set Enrichment Analysis (ssGSEA). Methylation levels were analyzed utilizing the UALCAN database. NPAS1-related mutations were analyzed using cBioPortal. Single-cell and tissue-specific expression of NPAS1 was examined using the Human Protein Atlas. Protein-protein interactions and transcription factor networks of NPAS1-associated genes were analyzed with STRING and Network Analyst.

**Results:** Our study found that the expression level of NPAS1 was higher in normal tissues compared to COAD tissues, higher NPAS1 expression was associated with better survival outcomes. The bioinformatics analysis confirmed that NPAS1 co-expressed genes were linked to diverse signalling pathways and cellular functions. NPAS1 is correlated with CD56bright, cytotoxic and NK cells, and negatively correlated with helper T cells and Tcm cells. The DNA methylation level of NPAS1 in tumor tissues was elevated compared to normal tissues. We analyzed the mutation characteristics of NPAS1, single-cell expression profiling of NPAS1, and protein-protein interactions involving genes associated with NPAS1. These discoveries offered perspectives on the etiology, identification, and management of COAD.

**Conclusion:** This study revealed a significant decrease of NPAS1 expression in COAD tissues, exhibiting associations with the clinical stage, prognosis, immune infiltration, and DNA methylation of COAD.

**Keywords:** colorectal adenocarcinoma, NPAS1, prognosis, immune infiltration, DNA methylation, gene mutation, single-cell RNAseq

## Background

Colorectal cancer stands as a formidable global health challenge, with yearly diagnoses comprising almost one-tenth of all cancer cases and cancer-related deaths across the globe.<sup>1</sup> It is one of the most prevalent malignancies affecting the gastrointestinal tract worldwide, with men ranking it third and women ranking it second.<sup>2</sup> Projections for 2035 are alarming, with the number of new colorectal cancer cases predicted to reach 2.5 million globally.<sup>3</sup> Within this landscape, urgent intervention is needed for Colorectal adenocarcinoma (COAD), which is the main subtype.<sup>4</sup> Current treatments for colorectal cancer encompass chemotherapy, surgery, and radiotherapy, the associated side effects and potential complications often impact patients' quality of life. Therefore, active prevention is essential. An in-depth study of the molecular mechanisms that underlie COAD development could pave the way for innovative therapeutic strategies. By illuminating these pathways, we may uncover novel targets and approaches that could revolutionize the treatment landscape for COAD, offering more effective and less invasive options for patients.

Genetic markers play a pivotal role in elucidating the mechanisms of tumorigenesis. Through targeted regulation of these markers, it becomes feasible to develop therapeutic strategies for primary tumor management and to inhibit the metastasis of cancer cells, thereby enhancing patient prognosis and survival rates.

Neuronal PAS domain protein 1 (NPAS1) is a protein coding gene that plays a crucial role in neuronal differentiation and the development of the nervous system,<sup>5</sup> it was suggested to play a protective or regulatory role during embryonic development.<sup>6</sup> Although the importance of NPAS1 in the nervous system is well established, its role in cancer remains to be fully explored. A study demonstrated that the incidence of homozygous deletions and single-copy variations of NPAS1 is markedly elevated in breast cancer patients compared to healthy females, indicating a potential critical involvement of NPAS1 in the development and progression of breast cancer.<sup>7</sup> Research indicates the existence of a complex bidirectional communication pathway between the gut and the brain, mediated by the nervous system, the vagus nerve, and neurotransmitters.<sup>8</sup> This gut-brain axis may provide valuable insights for further investigation into the role of NPAS1 in gastrointestinal function. However, research on NPAS1 in COAD remains unexplored, and this knowledge gap presents a significant opportunity for future studies to elucidate its potential implications in COAD.

A wide range of platforms and databases enables bioinformatic analysis of cancer data. By utilizing these resources, we integrated statistical and bioinformatics approaches to elucidate the role of the NPAS1 gene in COAD. This study aimed to assess the potential of NPAS1 as a diagnostic marker, explore its clinical relevance, and investigate its prognostic significance in order to gain a better understanding of COAD.

## Materials and Methods

### UALCAN Database Analysis

The UALCAN web portal (<http://ualcan.path.uab.edu>) offers a range of cancer types from the TCGA database, enabling users to explore an interactive network of resources and analyze the relative transcriptional gene expression between tumor and normal samples.<sup>9</sup> Utilizing UALCAN, we characterized the expression levels of the NPAS1 gene across various cancer types. Our study was centered on COAD, where we investigated the expression of NPAS1 and its association with important clinicopathological factors. Additionally, we examined the association between the levels of NPAS1 expression and the status of methylation.

### GEO and TCGA Cohorts

The gene expression profile matrix files of GSE44076 (98 normal adjacent tissues, 98 COAD tissues and 50 healthy colon tissues) and GSE74602 (30 normal adjacent tissues and 30 COAD tissues) gene expression profile matrix files were obtained from the GEO database (<https://www.ncbi.nlm.nih.gov/geo>).<sup>10</sup> These data were analyzed through comparison and visualized in charts (R package: ggplot2, R package: ggbeeswarm, Statistical method: Welch one-way ANOVA). In R software (version: 3.6.3), the Impute and Limma packages (version: 3.42.2) were used to perform background correction, normalization and log2 transformation on the matrix data of the GEO dataset. Using TCGA (<https://portal.gdc.cancer.gov>),<sup>11</sup> standardized RNA-seq data transcripts per million (TPM) and corresponding clinical characteristics of COAD patients were obtained for subsequent functional and immune analysis.

## Survival Analysis

The Kaplan–Meier plotter (<https://kmplot.com/analysis/>) was employed for examining the relationship among NPAS1 expression in TCGA samples and overall survival (OS) in COAD patients. Subsequently, the association among NPAS1 expression and overall survival in COAD patients with different clinical characteristics was analyzed. R (package: pROC) was utilized for the analysis and visualization of the receiver operating characteristic curve (ROC) associated with NPAS1 in COAD.

## Immune Cell Infiltration

We utilized the ssGSEA approach to systematically examine the levels of infiltration for 24 unique immune cell types in COAD. We then conducted gene expression profiling on each COAD sample, taking into account the attributes of the immune cells, thereby quantifying the enrichment score of each immune cell.<sup>12,13</sup> The immuno-invasiveness of NPAS1 in different tumors was analyzed using TISIDB (<http://cis.hku.hk/TISIDB>).<sup>14</sup>

## Enrichment Analysis

We conducted a GO analysis using the EnrichGO function (R package: clusterProfiler), followed by a KEGG analysis using the EnrichKEGG function (R package: clusterProfiler). Additionally, we performed GSEA utilizing the gseGO, gseKEGG, and gsePathway functions (R package: clusterProfiler).<sup>15</sup>

## DNA Methylation Analysis

We analyzed methylation level of gene through the MethSurv database, which is an internet-based tool utilized for conducting multivariate survival analysis by utilizing DNA methylation data (<https://biit.cs.ut.ee/methsurv/>). Individual CpGs in genes were clustered and analyzed in the form of heat maps using the MethSurv database. UALCAN was employed to assess the methylation levels of NPAS1.

## cBioPortal Database Analysis

cBioPortal (<https://www.cbioportal.org/>) website is an extensive database that serves as a comprehensive tool for the retrieval, download, analysis, and visualization of cancer genomics data.<sup>16</sup> It encompasses a wide array of genomic data types, consisting of mRNA and microRNA (miRNA) expression profiles, somatic mutations, DNA copy number alterations (CNAs), DNA methylation patterns, as well as protein and phosphoprotein abundance levels. cBioPortal can perform a variety of analyses, but the main ones are mutations related and their visualization. We conducted a genetic analysis of NPAS1 in samples of COAD obtained from the cBioPortal database.

## Single-Cell RNA-Seq Analysis

The Human Protein Atlas (<https://www.proteinatlas.org/>)<sup>17</sup> is a website where omics technology is used to map all human proteins present in cells, tissues, and organs. By searching for NPAS1 in the search box on this website, we explored its expression in different single-cell types and tissues, including colon tissues.

## PPI Network Enrichment Analysis

The STRING website (<https://string-db.org/>)<sup>18</sup> is a database for searching the interactions between known proteins and predicted proteins, and studying the interaction network between proteins helps to mine the core regulatory genes. NetworkAnalyst (<https://www.networkanalyst.ca/>)<sup>19</sup> is a visual online analysis platform for gene expression and meta-analysis, which can perform differential analysis, functional analysis and network analysis. We analyzed the related genes including NPAS1 by using the STRING database and set the interaction threshold to 0.70 to construct a protein-protein interaction (PPI) network. We used the Cytoscape tool and the NetworkAnalyst database to build circular network diagrams.

## COAD Tissues Collection

From March 2023 to January 2024, we obtained a total of 10 pairs of colon adenocarcinoma and adjacent normal tissues from individuals who underwent surgical removal for colon cancer at the Huangyan Hospital of Wenzhou Medical

University ([Supplementary Table 1](#)). Prior to surgery, these patients did not receive chemotherapy or radiotherapy. Samples were obtained from individuals following the acquisition of informed consent and with the endorsement of Ethics Committee of the Huangyan Hospital of Wenzhou Medical University (2021-KY037-02). Then, we used these 10 samples for WB and IHC staining and presented 10 sets of WB ([Supplementary Table 1](#), Number 1–10) results and 1 set of IHC result ([Supplementary Table 1](#), Number 1).

## Western Blot (WB) Assay

WB was performed according to our previous publications,<sup>20</sup> the experimental methods, reagents, and equipment are as follows: Briefly, cellular protein extracts processed with RIPA lysis buffer supplemented with benzoyl fluoride and a cocktail of protein phosphatase inhibitors. Protein concentrations were determined using a BCA protein quantification kit. The protein samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with 5% skim milk at room temperature for one hour to minimize any potential non-specific interactions. Subsequently, it was incubated with primary antibodies specific to the target proteins at 4°C overnight. After the washing process, the membrane was subsequently subjected to an hour-long incubation at room temperature with secondary antibodies conjugated with horseradish peroxidase (HRP). Detection of protein bands was achieved using a Western ECL substrate and visualized with the ChemiDox™ XRS+ system (BioRad). The proteins that were the focus of this study were specifically recognized by the primary antibodies employed: GAPDH (Proteintech; 60004-1-Ig) (Wuhan, China) (1:5000), NPAS1 (proteintech; 13701-1-AP) (Wuhan, China) (1:500). The original results of WB were presented in [Supplementary Figure 1](#).

## Immunohistochemistry (IHC) Staining

COAD and normal adjacent tissues were trimmed with paraffin embedding, sliced and incubated with 80% methanol for 10 min to block endogenous peroxidase activity. PBS cleaning, 0.1 mmol/L citrate buffer for antigen repair; After washing with PBS, 50 µL primary antibody (NPAS1, proteintech; 13701-1-AP) (Wuhan, China) (1:500) was added and incubated at 37 °C for 1 h. Washed with PBS, 50 µL of horseradish peroxidase labeled secondary antibody was added and incubated at 37 °C for 1 h; Washed with PBS and incubated with streptavidin-peroxidase at 37 °C for 1 h. After cleaning with PBS and redyeing with DAB, the film was sealed and observed under microscope. NPAS1 antibody is mainly located in the cytoplasm of tumor cells and is brown-yellow granular after staining.

## Statistical Analysis

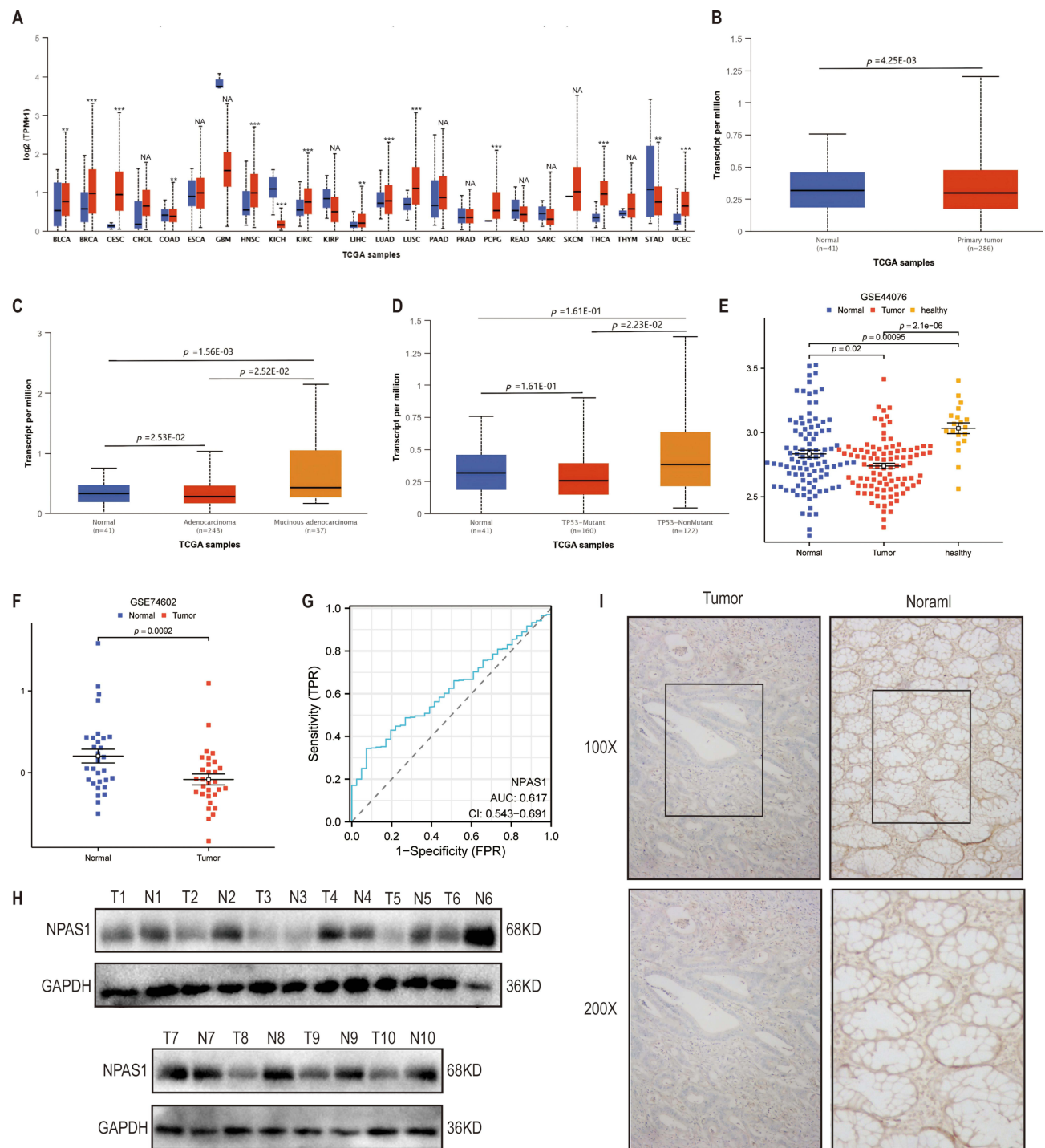
The R package (version 3.6.3) was used for statistical analysis and plotting. The Wilcoxon rank sum test and the Wilcoxon signed rank test were respectively applied. In all studies, a p value < 0.05 was defined as statistically significant. Background correction, normalization, and log2 conversion were performed using R software.

## Results

### The Expression of NPAS1 in COAD Tissues Was Decreased

Based on the research findings of NPAS1 in breast cancer, we initiated an investigation into its expression in COAD. We initially conducted a differential expression analysis of NPAS1 in 24 different cancer types, encompassing both primary tumor tissues and their corresponding normal tissues, utilizing the UALCAN database. The findings revealed that NPAS1 exhibited varying levels of upregulation and downregulation across distinct cancer types ([Figure 1A](#)), with a significant downregulation observed specifically in COAD tissue (normal, n=41; tumor, n=286; p=4.52E-03) ([Figure 1B](#)). Here, in a histological subtype analysis, NPAS1 was downregulated in adenocarcinoma compared to normal and mucinous adenocarcinoma ([Figure 1C](#)). Meanwhile, based on TP53 mutation status, the expression of NPAS1 in TP53 mutant in COAD was downregulated ([Figure 1D](#)). Next, we conducted a thorough investigation and acquired two gene expression profiles from GSE44076 and GSE74602, providing additional confirmation for the mRNA expression level of NPAS1. The two GEO datasets in this study were shown in [Figure 1E](#) and [F](#), the COAD tissue exhibited a notable decrease in the





**Figure 1** The expression of NPAS1 in COAD tissues. **(A)** In 24 different cancer types in UALCAN database, the upregulation and downregulation level of NPAS1. \*\*p < 0.01, \*\*\*p < 0.001, NA p > 0.05. **(B)** The expression level of NPAS1 in COAD based on sample types. **(C)** The expression level of NPAS1 in COAD based on histological subtypes. **(D)** The expression level of NPAS1 in COAD based on TP53 mutant status. **(E)** The expression level of NPAS1 in COAD in GSE44076 gene expression profile. **(F)** The expression level of NPAS1 in COAD in GSE74602 gene expression profile. **(G)** The ROC curve was generated to assess the value of NPAS1 in identifying COAD tissues. **(H)** The expression level of NPAS1 in 10 pairs of COAD tissues and adjacent normal tissues was detected by WB. **(I)** The expression level of NPAS1 in a pair of COAD tissue and adjacent normal tissue was detected by IHC staining.

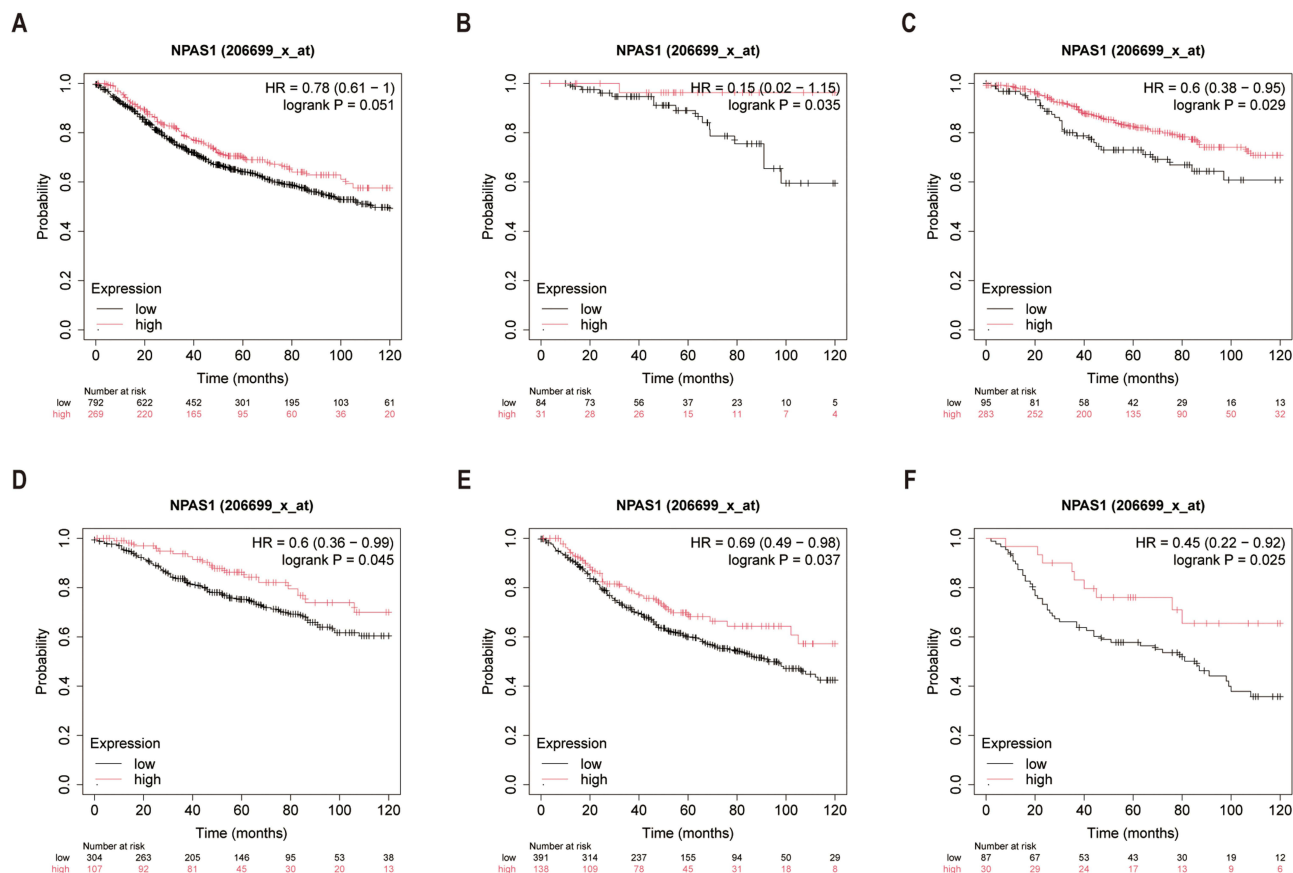
expression of NPAS1 compared to that observed in normal tissues. In addition, we set up the ROC curve, NPAS1 area under the curve (AUS) is 0.617 has certain diagnostic value (Figure 1G).

We subsequently employed Western blot analysis to assess the protein expression of NPAS1 in ten pairs of COAD tissues. Remarkably, we observed a significant decrease in its expression level compared to the corresponding normal adjacent tissues (Figure 1H). Additionally, IHC staining was performed to evaluate the differential expression of NPAS1, revealing a lower abundance of NPAS1 in COAD tissues as compared to normal adjacent tissues (Figure 1I).

## NPAS1 Is Associated with the Prognosis of COAD Patients

The data acquired from the Kaplan – Meier plotter was divided into two groups, namely low-risk and high-risk, using the median risk score as a criterion. The results showed COAD patients overexpressing NPAS1 had a better OS (HR=0.78, logrank P=0.051) (Figure 2A). Within the COAD subgroup, a positive correlation was observed between elevated NPAS1 expression and improved prognosis in the subsequent cases: pathologic stage I (HR=0.15, logrank P=0.035) (Figure 2B), pathologic stage II (HR=0.6, logrank P=0.029) (Figure 2C), stage N0 (HR=0.6, logrank P=0.045) (Figure 2D), female patients (HR=0.69, logrank P=0.037) (Figure 2E) and MSI-stable (HR=0.45, logrank P=0.025) (Figure 2F).

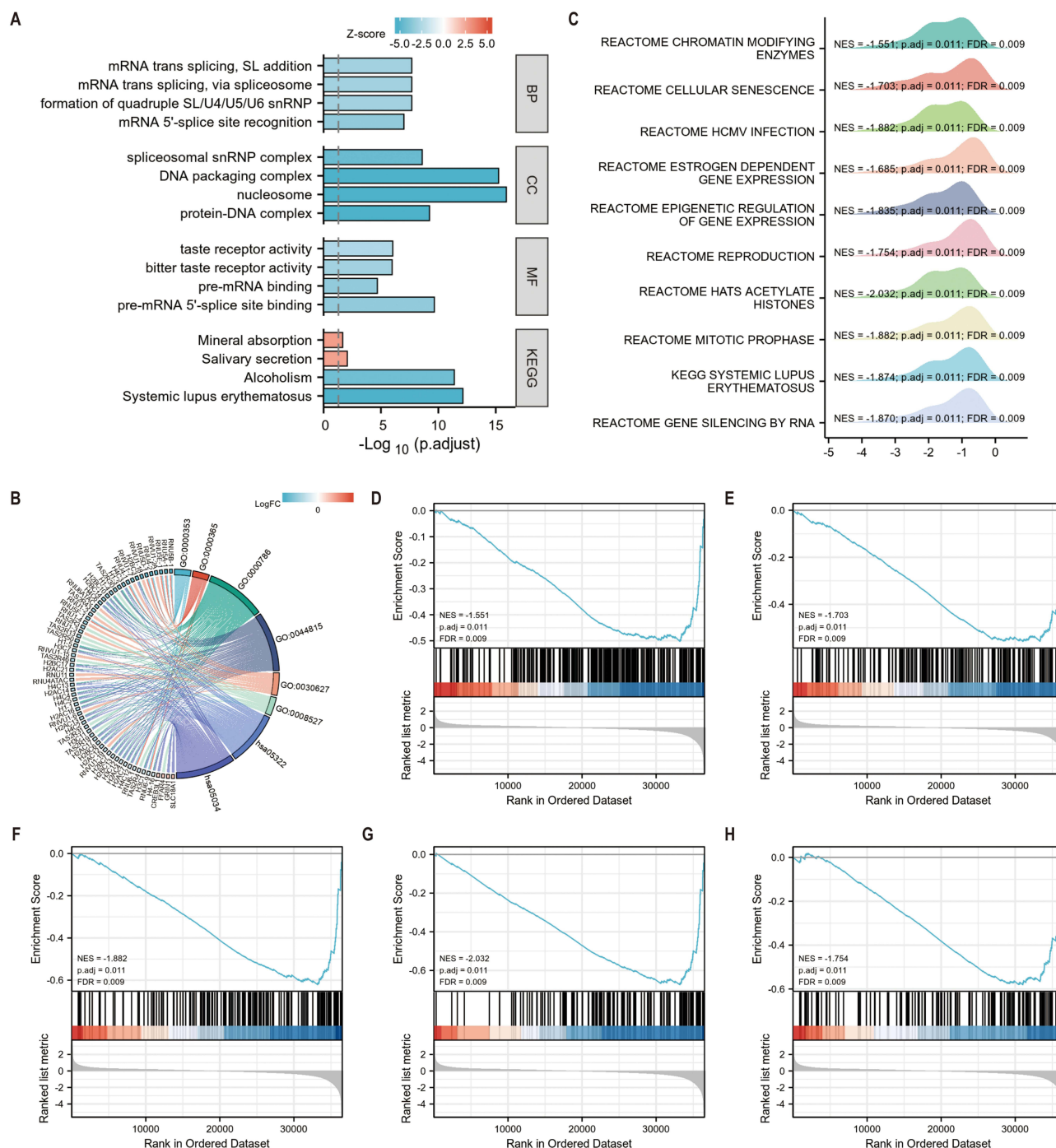
We also performed univariate analysis and multivariate analysis of pathologic T stage, pathologic N stage, pathologic M stage, gender, age, weight, height, BMI, histological type, CEA level and NPAS1 expression level to explore additional variables linked to varying results (Supplementary Table 2). In univariate analysis, NPAS1 expression was associated with OS (Supplementary Table 3).



**Figure 2** Kaplan-Meier survival plots were used to compare the low and high expression of NPAS1 in COAD. (A) OS survival curve of patients with COAD between high and low expression of NPAS1. (B and C) OS survival curves of stage I and stage II between NPAS1-high and -low patients with COAD. (D) OS survival curve of stage N0 between NPAS1-high and -low patients with COAD. (E) OS survival curve of female between NPAS1-high and -low patients with COAD. (F) OS survival curve of MSI stable between NPAS1-high and -low patients with COAD.

## GO and KEGG Enrichment Analysis of NPASI-Related Genes

To investigate the correlation between NPASI-related genes and their biological functions, we utilized TCGA data for conducting an analysis. The results of GO analysis indicated that NPASI-related genes are mainly concentrated in the regulation of mRNA trans splicing in biological process (BP), nucleosome and DNA packaging complex in cellular composition (CC), pre-mRNA 5'-splice site binding in molecular function (MF). In the KEGG enrichment analysis, alcoholism and systemic lupus erythematosus were found to be the primary associations of NPASI-related genes (Figure 3A and B).



**Figure 3** Enrichment of biofunction and associated genes analysis of NPASI in COAD. (A and B) The GKEGG analysis of NPASI. (C-H) The GSEA analysis of NPASI.

## NPAS1-Related Signaling Pathways Based on GSEA

To explore NPAS1-Related Signaling Pathways in COAD, we conducted a GSEA utilizing gene sets sourced from the Molecular Signatures Database Collection (MSigDB) (c2.cp.reactome/biocompare/kegg.v6.2.symbols.gmt). The enrichment plots of the GSEA showed that genes were enriched in differentially down-regulated genes and NPAS1 was down-regulated in several signaling pathways, including chromatin modifying enzymes, cellular senescence, hcmv infection, estrogen dependent gene expression, reproduction, hats acetylate histones, mitotic prophase, systemic lupus erythematosus and gene silencing by RNA (Figure 3C–H).

## Differentially Expressed Genes Between Individuals with High and Low Expression Levels of NPAS1

We categorized the data of COAD patients into two groups based on the varying levels of NPAS1 expression, allowing for a comparison between individuals with high and low NPAS1 expression. A total of 4010 genes exhibited differential expression in the NPAS1 high-low expression group, with 468 genes showing up-regulation and 3542 genes displaying down-regulation (Supplementary Figure 2A). A statistically significant result was defined as having an adjusted p value less than 0.05 and an absolute log2-fold change greater than 1. Subsequently, the correlation between NPAS1 and the leading 10 differentially expressed genes (DEGs) (including ANXA10, CLDN18, CSAG1, MAGEA12, FGL1, PSAPL1, INSL4, GP2, MUC5AC, AC105460.1) and NPAS1 was examined (Supplementary Figure 2B). The results indicated that NPAS1 was significantly correlated with ANXA10, CLDN18, MAGEA12, FGL1, PSAPL1, INSL4, GP2, and MUC5AC ( $p < 0.05$ ), suggesting potential interaction relationships.

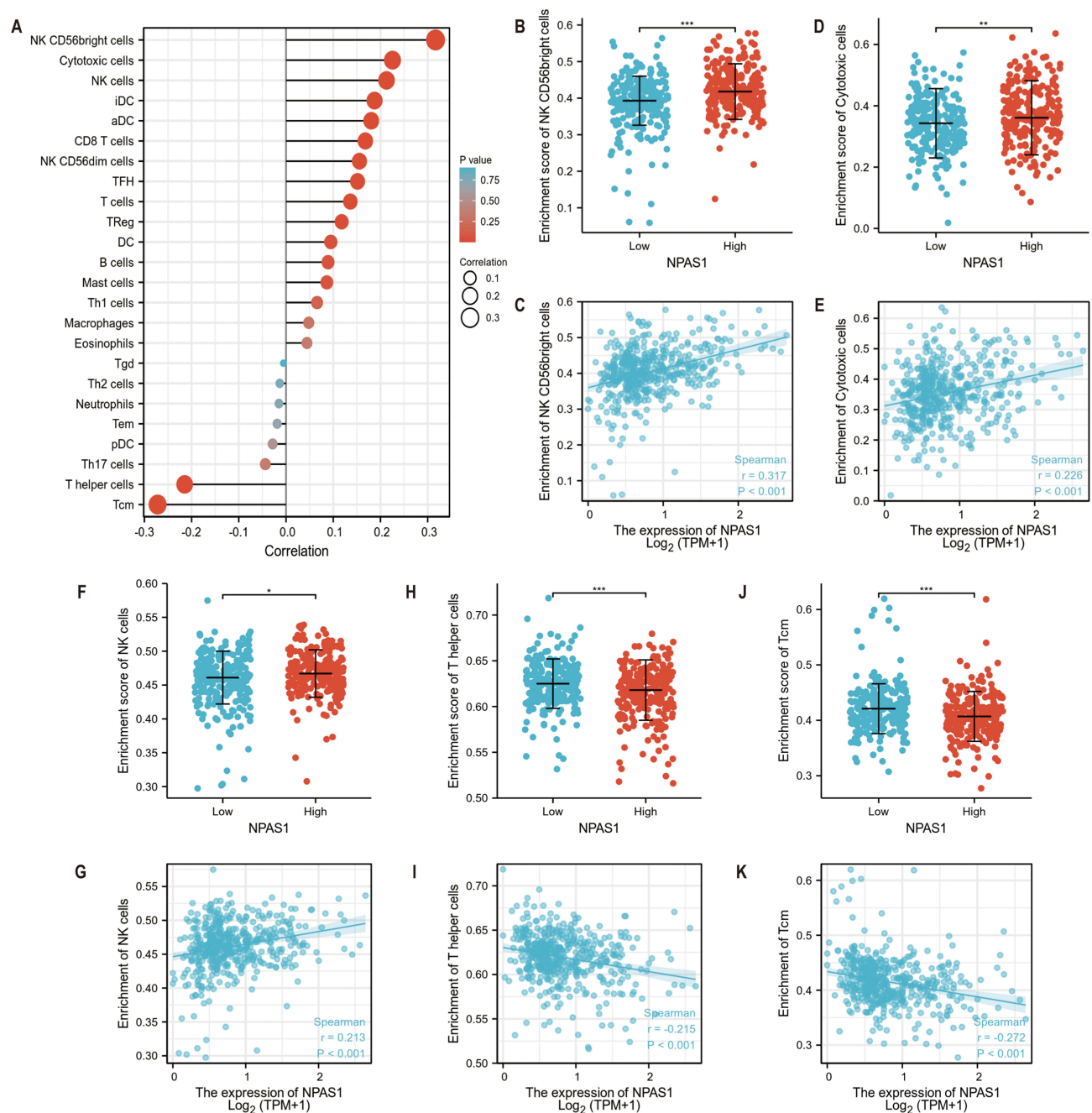
## Correlation Between NPAS1 Expression and Immune Characteristics

It is widely acknowledged that the presence and progression of tumors are significantly influenced by the regulatory function exerted by immune cells infiltrating into the tumor microenvironment,<sup>21</sup> we postulated that the potential involvement of NPAS1 in modulating the immune response to tumors. We utilized the ssGSEA algorithm to assess the associations between NPAS1 expression and the levels of infiltration by immune cells (Figure 4A). NPAS1 has positive correlations with CD56bright cells ( $R=0.317$ ,  $P<0.001$ ), cytotoxic cells ( $R=0.266$ ,  $P<0.001$ ), NK cells ( $R=0.213$ ,  $P<0.001$ ), and it was negatively correlated with T helper cells ( $R=-0.215$ ,  $P<0.001$ ) and Tcm cells ( $R=-0.272$ ,  $P<0.001$ ) (Figure 4B–K). Simultaneously, we employed the TISIDB database to examine the correlation between NPAS1 expression and immune cell infiltration levels across various tumor types (Supplementary Figure 2C). The results indicate that NPAS1 exhibits a significant positive correlation with CD56bright cells across multiple cancer types, including COAD. CD56 bright cells, as a subset of NK cells, can produce various cytokines and play a significant role in shaping the early immune response and adaptive response (IFN- $\gamma$ ), as well as in regulatory NK cells (IL-10).<sup>22</sup> NPAS1 may be involved in the regulation of immune responses mediated by natural killer cells.

## Correlation Between Methylation and NPAS1 Expression

To explore the underlying mechanism behind the reduced expression of NPAS1 in COAD, we employed online resources to examine the association between NPAS1 expression levels and its methylation status. To begin with, the promoter region of NPAS1 was subjected to DNA methylation analysis using the UALCAN online database. We noticed a notable increase in DNA methylation levels at the promoter of NPAS1 in COAD compared to normal tissue (Figure 5A), and this methylation was found to be strongly associated with cancer stage (Figure 5B), nodal metastasis status (Figure 5C), patient's age (Figure 5D) and TP53 mutation status (Figure 5E). Then, using the MethSurv database, we performed a cluster analysis of individual CPG in NPAS1 in the form of heat maps, establishing associations between methylation levels and patient characteristics as well as gene subregions (Figure 5F). Heat map methylation level (0=completely unmethylated; 1=complete methylation) is displayed as a continuous variable from blue to red. These findings may indicate that aberrant methylation underlies NPAS1 gene silencing and its reduced expression in COAD.



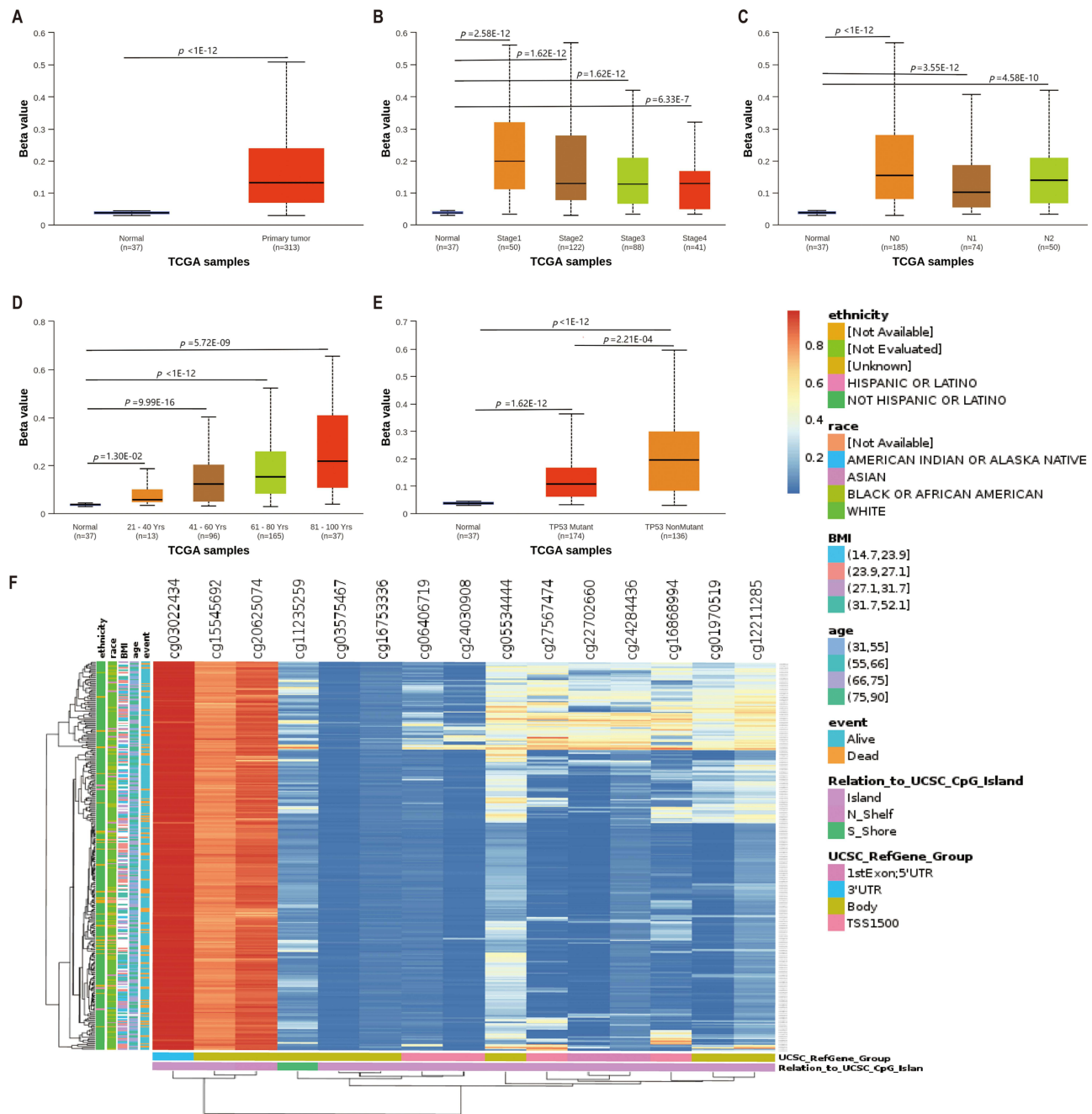


**Figure 4** Correlation between NPAS1 expression and immune characteristics. (A) Correlation between the expression level of NPAS1 and the proportions of 24 immune cell types. (B–K) Scatter plot of NPAS1 expression and individual immune cell infiltration levels, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## Genetic Analysis of NPAS1 Variants in COAD

We conducted a genetic analysis on NPAS1 alterations in COAD samples obtained from the cBioPortal database. According to the findings in the OncoPrint section, a total of 1602 COAD samples were analyzed for gene sequence and copy number variation data. Among these samples, 23 cases exhibited gene mutation, resulting in an overall mutation rate of 1.4%, including three cases of amplification, four cases of truncating mutation and 16 cases of missense mutation (Figure 6A). In addition, we analyzed specific mutation sites of NPAS1 and the relationship between mutations and different subtypes (including neoplasm disease stage, cancer type, tumor grade, race category and MSI status) (Figure 6B–G). The occurrence of NPAS1 changes in various datasets and cancer types, along with the proportions of mutation, amplification, and deep deletion components, were also illustrated (Figure 6H–J).

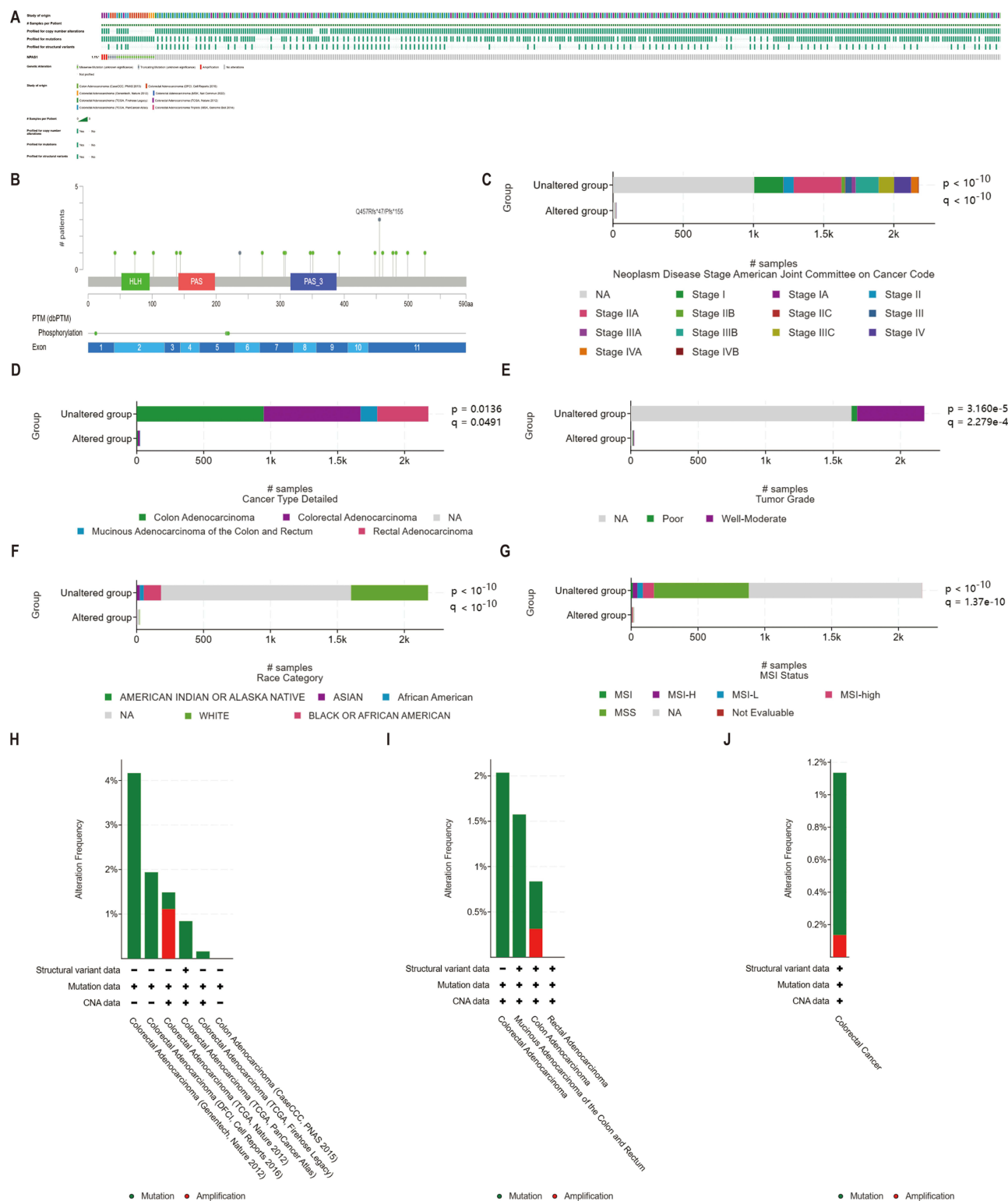




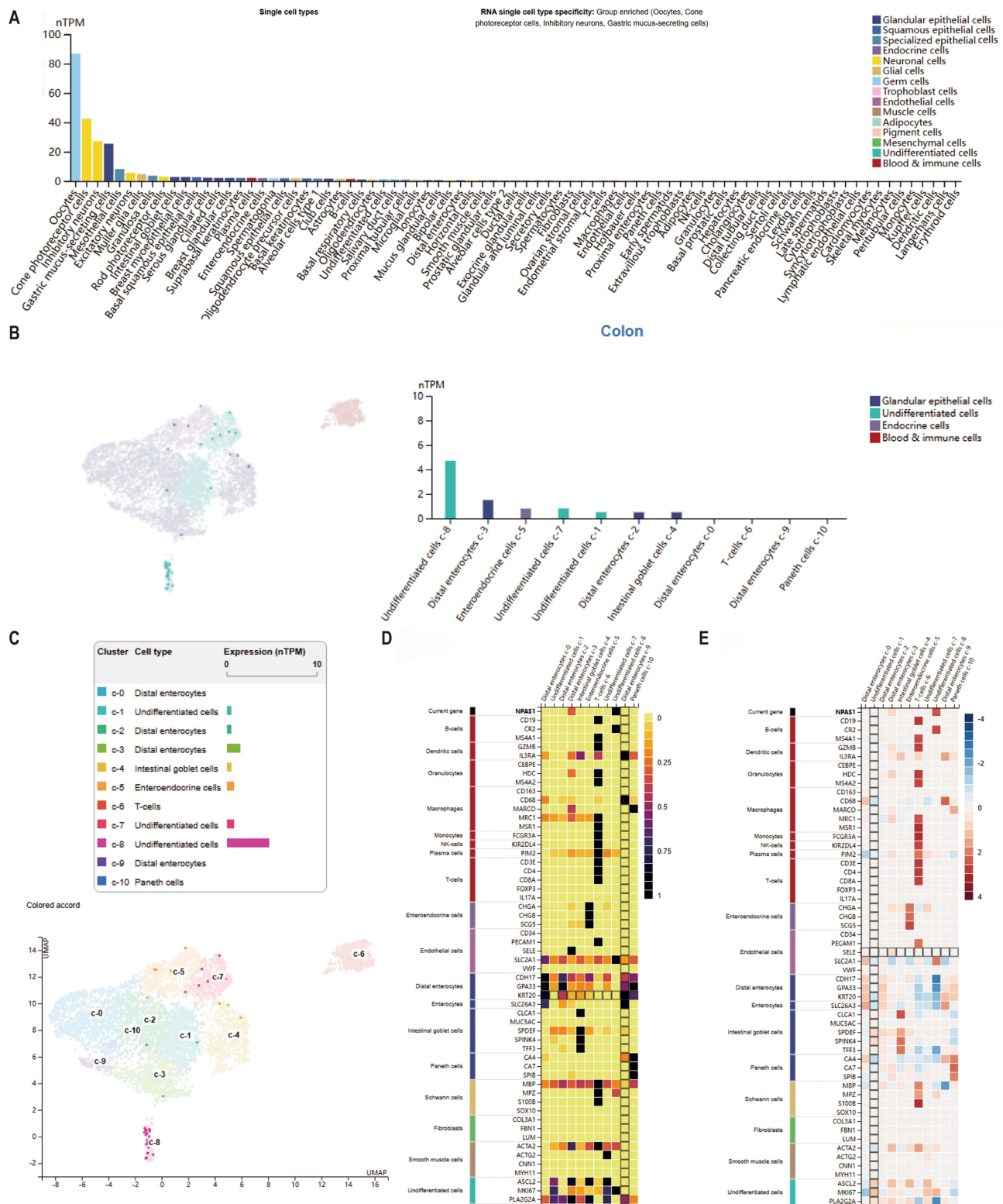
**Figure 5** DNA methylation of NPASI. **(A)** DNA methylation level of NPASI in COAD and normal tissues. **(B)** DNA methylation level of NPASI in COAD patients with different cancer stages. **(C)** DNA methylation level of NPASI in COAD patients with different nodal metastasis status. **(D)** DNA methylation level of NPASI in COAD patients with different ages. **(E)** DNA methylation level of NPASI in COAD patients with TP53 mutation status. **(F)** Individual CPG in NPASI methylation levels with available patient characteristics and gene subregions.

## Single-Cell RNA Sequencing Analysis of NPASI Expression in the Colon

The top five NPASI expression levels from high to low were oocytes, cone photoreceptor cells, inhibitory neurons, gastric mucus-secreting cells and mesothelial cells. NPASI is predominantly expressed in oocytes, with 86.8 standardized transcripts per 1 million protein-coding genes (nTPM). It was also expressed in intestinal goblet cells (2.8 nTPM), enteroendocrine cells (2.0 nTPM), undifferentiated cells (1.1 nTPM), distal enterocytes (0.6 nTPM), proximal enterocytes (0.1 nTPM) and paneth cells (0.1 nTPM) (Figure 7A). In the colon fraction, NPASI was expressed in the following order: undifferentiated cells (c-8, 4.7 nTPM), distal enterocytes (c-3, 1.4 nTPM), enteroendocrine cells (c-5,



**Figure 6** Alterations of NPASI in COAD. **(A)** Waterfall diagram illustrating the distribution and categorization of genetic mutations in NPASI gene in patients with COAD. **(B)** Specific mutation site of NPASI. **(C–G)** Mutation site of NPASI and the relationship between mutations and neoplasm disease stage, cancer type, tumor grade, race category and MSI status. **(H–J)** NPASI variants in different COAD datasets.



**Figure 7** Single-cell RNAseq analysis of NPAS1. **(A)** NPAS1 mRNA expression in various cell types. **(B and C)** The expression level of NPAS1 mRNA varies in different cell populations in the colon. **(D and E)** Expression of the NPAS1 and type markers in the various single-cell clusters of the colon, with Mas-norm method and Z-score method.

0.8 nTPM), undifferentiated cells (c-7, 0.8 nTPM; c-1, 0.5 nTPM), distal enterocytes (c-2, 0.5 nTPM), intestinal goblet cells (c-4, 0.5 nTPM) (Figure 7B). At the same time, each cell type was divided into distinct cell clusters based on the difference in NPAS1 nTPM expression levels (Figure 7C). Finally, using the statistical methods of Mas-norm and Z-score, heat maps were drawn to show the expression of NPAS1 and type markers in each single-cell cluster in the colon (Figure 7D and E).

## PPI Network of NPAS1-Associated Genes and the TF-Gene Interaction Network

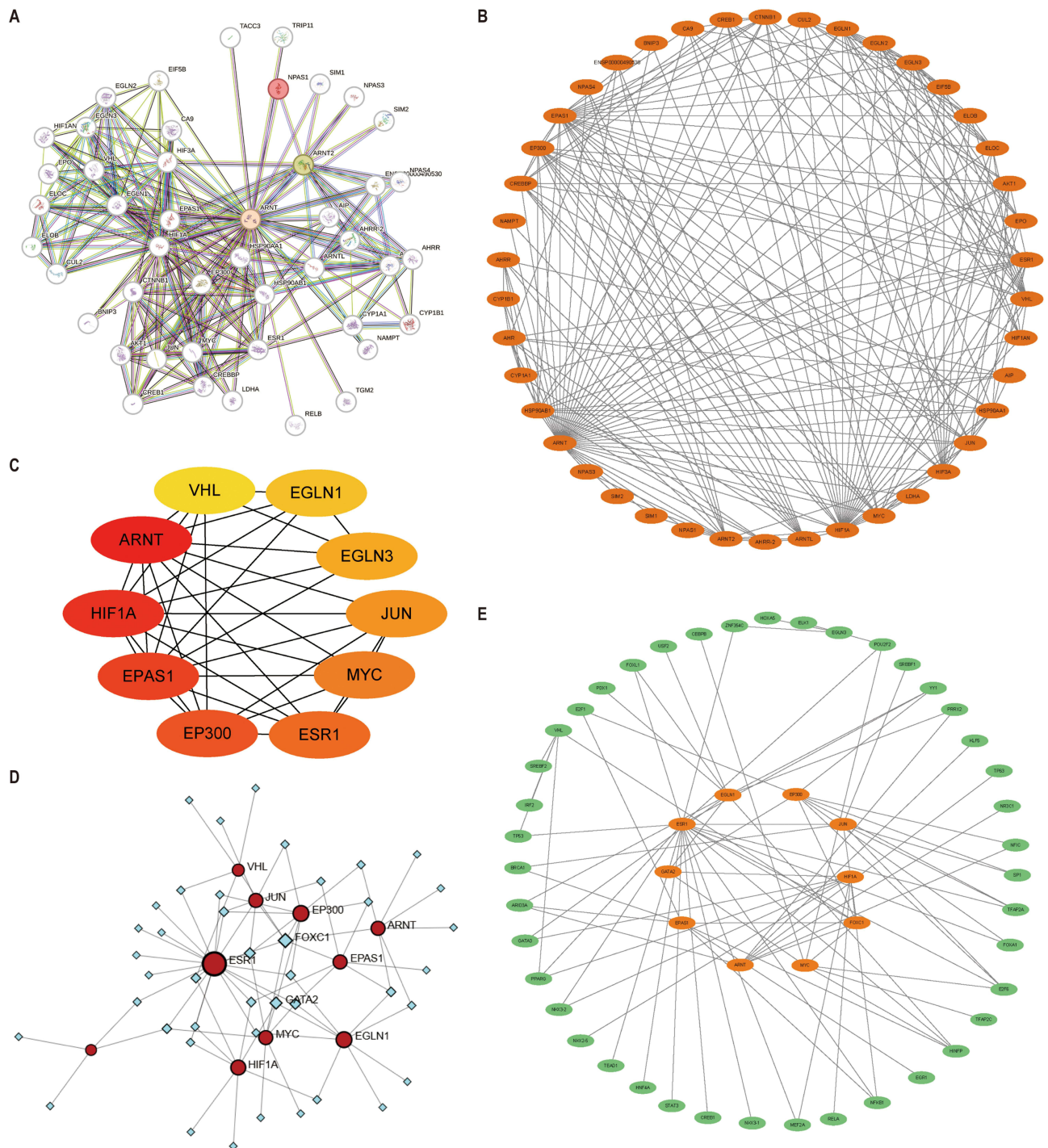
We constructed a PPI network by analyzing 221 associated genes, including NPAS1, using the STRING database with an interaction threshold of 0.70 (Figure 8A). Additionally, we utilized the Cytoscape tool to generate a circular network diagram (Figure 8B) and identified the top 10 hub genes, including ARNT, HIF1A, EPAS1, EP300, ESR1, MYC, JUN, EGLN3, EGLN1 and VHL (Figure 8C). Next, we predicted the transcription factor of these 10 core genes and constructed the TF-Gene interaction network through NetworkAnalyst database (Figure 8D) and then generated the circular network diagram through Cytoscape tool (Figure 8E).

## Discussion

COAD stands as a prominent contributor to global cancer mortality and poses significant challenges in terms of treatment, given its elevated fatality rate during advanced stages.<sup>23,24</sup> relevant clinical research has indicated that the approximate survival rate for patients diagnosed with COAD over a period of five years is around 56%.<sup>25</sup> Both genetic and environmental factors contribute to the onset of COAD. Approximately 10–20% of individuals diagnosed with colorectal cancer have a familial predisposition.<sup>26</sup> Some lifestyle factors increase the risk of colorectal cancer, such as excessive alcohol consumption,<sup>27</sup> smoking,<sup>28</sup> obesity,<sup>29</sup> gut dysbiosis, gut microbiota<sup>30</sup> and infection with specific bacterial species, such as *fusobacterium nucleatum* and *bacteroides fragilis*,<sup>31–33</sup> may all increase the risk of colorectal cancer. Colonoscopy is the most commonly used diagnostic technique for identifying colorectal cancer, as it effectively identifies advanced lesions; however, early stages of colorectal cancer may manifest as inconspicuous mucosal abnormalities that pose challenges in detection.<sup>34</sup> Nowadays, numerous genes associated with tumors have been recognized as prognostic biomarkers for early detection and hold immense importance in the advancement of tumor treatment. Therefore, potential prognostic molecular biomarkers are particularly needed to improve the prognosis of COAD patients and provide more precise clinical interventions.

NPAS1, a member of the neuronal PAS (Per-Arnt-Sim) domain transcription factor (TF) family, is a transcription factor known as a basic helix-loop-helix-PAS, which is expressed in the central nervous system and believed to play a role in promoting neuronal differentiation.<sup>35</sup> Studies of the relevant mouse genes have shown that NPAS1 plays a role in neurons, and the precise role of the gene remains elusive, and it is speculated to potentially contribute to safeguarding or governing processes in the later stages of embryogenesis and postnatal maturation.<sup>6</sup> NPAS1 has been reported to be a major regulatory factor of risk genes associated with neuropsychiatric disorders. It exerts negative regulation on the promoter of tyrosine hydroxylase through the formation of a dimer with aryl hydrocarbon receptor nuclear translocator (ARNT), thereby modulating the level of tyrosine hydroxylase in dopaminergic neurons and contributing to the pathogenesis of diseases such as Parkinson's disease. Moreover, NPAS1 regulates the expression of brain - derived erythropoietin in response to cellular oxygen levels by binding to the enhancer region of the erythropoietin gene and suppressing hypoxia response element - mediated gene expression.<sup>36–38</sup> The prevalence of NPAS1 deletions, including homozygous deletions and single-copy variants, was found to be higher in breast cancer patients compared to healthy women in a study,<sup>7</sup> the alterations in NPAS1 copy number may exert a significant impact on the development of breast cancer. However, there is limited knowledge regarding the molecular mechanism underlying NPAS1's involvement in COAD. The complex bidirectional communication between the gut and the brain is mediated through the enteric nervous system, the vagus nerve, and various neurotransmitters. In the colon, this interaction involves multiple neuronal proteins and neurons, including sensory neurons that detect mechanical stimuli and transmit signals to the central nervous system, as well as the enteric nervous system, which regulates intestinal motility and secretion. The gut's resident microbiota also plays a role by producing substances like serotonin and influencing the gut-brain axis.<sup>8,39</sup> This interconnected pathway may provide a mechanistic basis for hypothesizing an association between NPAS1 and COAD. NPAS1 may play





**Figure 8** PPI Network Analysis. **(A)** PPI network diagram of NPAS1-associated genes. **(B)** Circular network diagram of NPAS1-associated genes. **(C)** Top ten Hub genes in the PPI network. **(D)** TF-Gene interaction network diagram constructed by the transcription factors of the top ten Hub genes. **(E)** Circular network diagram constructed by the transcription factors of the top ten Hub genes.

a significant role in vagal sensory neurons or intrinsic intestinal neurons. Deficiency or mutation of NPAS1 may modify the sensitivity of neurons to intestinal signals, consequently impacting gastrointestinal function. Moreover, the expression of NPAS1 may be regulated by microbial metabolites, which together influence the integrity of the intestinal barrier and local immune responses. These hypotheses will be further investigated in our subsequent experiments.

In this study, through the mining and comprehensive analysis of the TCGA dataset, we observed a strong association between the presence of NPAS1 and the occurrence, development, prognosis, immune infiltration and DNA methylation level of COAD.

Through analysis of the UALCAN database, we found that anomalous expression of NPAS1 has been observed in numerous cancer types and significantly reduced in COAD and associated with histological subtype of COAD and TP53 mutation status. We also searched two GEO datasets to further validate the mRNA expression level of NPAS1 and obtained consistent results. We subsequently examined the presence of NPAS1 in both colon adenocarcinoma and the normal adjacent tissues of patients through WB and IHC staining, the findings indicated a notable decrease in the protein expression level of NPAS1 in COAD tissues compared to normal adjacent tissues. To investigate the significance of NPAS1 in COAD, we examined the correlation between NPAS1 expression and the prognosis of patients with COAD using data from the Kaplan-Meier plotter database. COAD patients with overexpression of NPAS1 have better OS, while cases with high expression of NPAS1 and good prognosis of COAD include pathologic stage I, pathologic stage II, stage N0, MSI-stable and female patients.

Through the analysis of biological function correlation, we can understand the biological function and related pathways of NPAS1-related genes. GO analysis showed that NPAS1-related genes mainly play a role in regulating mRNA trans splicing, nucleosome, DNA packaging complex and pre-mRNA 5'-splice site binding, and KEGG showed that NPAS1-related genes were associated with alcoholism and systemic lupus erythematosus. GSEA enrichment analysis showed that NPAS1 is downregulated in signaling pathways such as chromatin modifying enzymes, cellular senescence, hcmv infection, estrogen dependent gene expression, reproduction, hats acetylate histones, mitotic prophase, systemic lupus erythematosus and gene silencing by RNA. At the same time, we analyzed the differentially expressed genes of the high expression group of NPAS1 and the low expression group and statistically analyzed the correlation between the top 10 DEGs and NPAS1.

The understanding of the immune system's involvement in cancer has been established for a considerable period. Attention has begun to be paid to the correlation between the infiltration of immune cells and the prognosis and treatment response of tumors, as well as the potential to reinforce traditional cancer therapy by integrating active immunotherapy through the utilization of therapeutic cancer vaccines.<sup>40</sup> Tumor immunity is likely to become a significant and emerging area in the field of cancer treatment. The cytotoxic function of NK cells, including its two major subsets CD56bright and CD56dim NK cells, has emerged as a novel therapeutic target for tumor therapy. The presence of NK cells has been consistently linked to enhanced overall survival rates across a diverse range of tumor types.<sup>41</sup> In this study, we investigated the association between the expression of NPAS1 and the extent of infiltration by immune cells. The findings indicated a positive association between NPAS1 and CD56bright cells, cytotoxic cells, NK cells. On the contrary, T helper cells and Tcm cells showed a negative correlation. The results may provide new insights into the role of NK cells in improving survival in COAD.

DNA methylation is a widely recognized epigenetic alteration that guarantees the appropriate control of gene expression and enduring suppression of genes in healthy cells.<sup>42</sup> It is well known that the inactivation of many tumor suppressor genes occurs due to hypermethylation within the promoter region, and there exists a broad spectrum of genes in various cancer types where DNA methylation is suppressed. Through analysis of the UALCAN online database, we observed that the DNA methylation level of NPAS1 at the promoter in COAD was significantly higher than that in normal tissues, and the degree of methylation was significantly correlated with cancer stage, nodal metastasis status, patient's age and TP53 mutation status. It can be reasonably inferred that the cause behind the diminished NPAS1 expression in COAD may be the initiation of hypermethylation.

We conducted genetic analysis of NPAS1 alterations in COAD samples, including identification of specific mutation sites and their association with different subtypes. Additionally, we investigated the expression profile of NPAS1 using Single-cell RNAseq, revealing its predominant presence in oocytes as well as various colon cell types such as undifferentiated cells, distal enterocytes, enteroendocrine cells, intestinal goblet cells. Furthermore, a PPI network comprising 221 related genes was constructed to explore the interactions involving NPAS1. Finally, we predicted transcription factors for core genes and established a TF-Gene interaction network.

Although our research analyzed the potential role of NPAS1 in COAD through bioinformatics, there are still some limitations and deficiencies. Firstly, predicting prognosis through a single cohort in public datasets is far from perfect. The important regulatory mechanism of NPAS1 in COAD still needs to be further verified and evaluated by analyzing clinical samples from more centers. All types of clinical features should be included, including different treatment methods for patients, but some information is inevitably lost in public databases. Secondly, the molecular mechanism of NPAS1 in the occurrence and development of COAD has not been studied, such as the pathways through which NPAS1 regulates the progression of COAD, the mechanism of the correlation between NPAS1 and immune cells and more bioinformatics results regarding the colon microenvironment. In our subsequent research, we will design and implement relevant experiments.

## Conclusion

This study represents the first comprehensive analysis of the role of NPAS1 in COAD, demonstrating its expression and establishing a close association with clinical stage, prognosis, immune infiltration, and DNA methylation in COAD. Furthermore, we investigated NPAS1 mutation characteristics in COAD samples, examined its expression profile using single-cell RNAseq technology, constructed a protein-protein interaction network involving NPAS1-related genes, predicted transcription factors for core genes, and established a TF-Gene interaction network. These research endeavors aim to enhance our understanding of the significance of NPAS1 in oncology and emphasize its value as a prognostic predictor.

## Data Sharing Statement

These data of this manuscript can be available from the corresponding author Qigang Ye, upon reasonable requests.

## Ethics Approval and Consent to Participate

This study conformed to the guidelines of the Helsinki Declaration. Patients/participants in this study provided written informed consent to participate in this study. Studies involving human subjects were reviewed and approved by the Ethics Committee of the Huangyan Hospital of Wenzhou Medical University.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare no potential conflicts of interest regarding the research, authorship, or publication of this article.

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