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ORIGINAL RESEARCH

Downregulation of NAT1 Expression is Associated with Poor Prognosis and Immune Infiltration in COAD

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Background: An increasing corpus of evidence has identified the involvement of N-acetyltransferase 1 (NAT1), a member of the NAT family, in the progression of various cancers. However, the specific function of NAT1 in colon cancer (COAD) remains elusive. This study aims to decip her the role of NAT1 in COAD and its associated mechanisms.

Methods: The Tumor Immunity Evaluation Resource (TIMER), The Cancer Genome Atlas (TCGA), and the Gene Expression Omnibus (GEO) databases were employed to assess the NAT1 expression level in COAD. The differential expression between COAD and normal colon tissue was further validated using quantitative real-time reverse-transcription PCR (RT-qPCR) and Western blot (WB) analyses. Additionally, survival analysis of NAT1 in COAD was carried out using the PrognoScan database and TCGA dataset. The functions of NAT1 were explored through gene set enrichment analysis (GSEA) and immuno-infiltration analysis.

Results: There was a significant reduction in NAT1 expression in COAD samples compared to normal tissue. Notably, low NAT1 expression in COAD correlated significantly with various clinical parameters such as tumor stage (T stage, N stage, M stage, pathologic stage), primary therapy outcome, carcinoembryonic antigen (CEA) level, and lymphatic invasion. The downregulation of NAT1 was also strongly linked with poor outcomes in overall survival (OS), progression-free interval (PFI), and disease-specific survival (DSS). Cox regression analysis highlighted NAT1 as an independent prognostic indicator for overall survival in COAD patients. GSEA results revealed NAT1's involvement in multiple pathways, including the neuroactive ligand-receptor interaction, olfactory transduction, olfactory signaling, extracellular matrix receptor interaction, calcium signaling, and focal adhesion pathways. Furthermore, NAT1 expression was found to significantly correlate with infiltration levels of various immune cells.

Conclusion: The findings reveal NAT1's potential as a valuable prognostic biomarker for COAD. Moreover, its associated mechanisms offer insights that might pave the way for therapeutic interventions for COAD patients.

Keywords: NAT1, COAD, prognosis, immune infiltration, RT-qPCR, western blot

Introduction

Colon cancer (COAD) is a prevalent malignant tumor of global significance, characterized by high incidence and mortality rates.[1] As per the 2020 statistics, the worldwide incidence of COAD exceeded 1.14 million new cases, resulting in approximately 570,000 deaths, which respectively contributed to 6% and 5.8% of the total new cancer cases and mortalities globally.[2] The American Cancer Society's forecast data predicts that by 2023, the United States alone will witness approximately 100,000 new cases of COAD.[3] Concurrently, in China, the incidence and mortality rates of COAD are experiencing an annual escalation.[4] The present therapeutic modalities for COAD comprise surgical intervention, radiotherapy, chemotherapy, targeted therapy, and immunotherapy.[5] Despite noteworthy progress in the diagnosis and management of colon cancer, the prognosis for advanced-

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stage (Stage III and IV) tumors remains disconcertingly unfavorable.[6] In recent years, studies on the pathogenesis and treatment for COAD have garnered substantial interest. Nevertheless, the current treatment outcomes are still suboptimal, and efficacious measures to significantly curtail the mortality rates among COAD patients are conspicuously deficient. Thus, it is imperative to undertake an in-depth exploration of the molecular mechanisms to identify potential screening biomarkers and therapeutic targets for COAD.

The N-acetyltransferase (NAT) family is a group of catalytic enzymes that includes multiple N-acetyltransferases.[7] These enzymes facilitate acetylation reactions in cellular metabolic processes by transferring acetyl groups to substrate molecules' amino or hydroxyl groups, thereby regulating their bioactivity. The NAT family is primarily composed of two subtypes, NAT1 and NAT2, which are expressed in various tissues throughout the human body and play essential roles in physiological processes such as drug metabolism, liver detoxification, and cell apoptosis.[8] The NAT family's aberrant expression and functional dysregulation have been established as closely linked to the development and advancement of multiple diseases, such as cancers, autoimmune diseases, and neurodegenerative diseases.[9,10] Notably, NAT1 is of significant importance as a member of the NAT family, as it is involved in biological processes such as drug metabolism and biosynthesis. Moreover, its differential expression and polymorphisms may be associated with the onset of various cancers, including breast cancer, bladder cancer, and prostate cancer.[11–13] Several studies have investigated the involvement of NAT1 in breast cancer. Endo et al discovered that miR-1290 can regulate breast cancer growth and metastasis by targeting the 3'-UTR region of the NAT1 gene.[14] Additionally, the expression of NAT1 is significantly associated with the prognosis of breast cancer patients, particularly those with lymph node-positive disease. Hein et al suggest that NAT1*10 may have protective effects, while the slow acetylation genotype may increase the risk of breast cancer.[15] Notwithstanding our extensive comprehension of the involvement of NAT1 in breast cancer, its biological and prognostic significance in COAD remains unclear. This lacuna in knowledge necessitates immediate and comprehensive research for elucidation.

In this study, we systematically analyzed the correlation between NAT1 expression and clinicopathological features in colon cancer patients, utilizing the Tumor Immunity Evaluation Resource (TIMER), The Cancer Genome Atlas (TCGA), and Gene Expression Omnibus (GEO) databases. The diagnostic and prognostic significance of NAT1 in colon cancer was assessed, and the potential mechanism of NAT1 in colon cancer pathogenesis and progression was thoroughly investigated through Gene set enrichment analysis (GSEA). Furthermore, the correlation between NAT1 expression and immune cell infiltration in the tumor microenvironment of COAD was also investigated. This investigation not only underscores the significance of NAT1 in colon cancer but also suggests that NAT1 may be a prognostic biomarker and therapeutic target in colon cancer.

Material and Methods

TIMER Database Analysis

TIMER is an online resource platform dedicated to the analysis of immune cell infiltration and expression levels of specific genes in different cancer types.[16] Using the TIMER database, we investigated the differences in the expression of NAT1 between cancer and normal tissues of various cancer types.

TCGA Data Download and Analysis

TCGA is a research project aimed at accelerating the understanding of tumor biology through large-scale genome sequencing analysis of different types of cancer.[17] In this study, we procured RNA-seq sequencing data and associated clinicopathological data for 478 colon cancer tissues and 41 normal colon tissues from the TCGA database. Table 1 presents the primary clinicopathological characteristics of COAD patients. Furthermore, to authenticate the expression of NAT1 mRNA in colon cancer patients, we downloaded the gene expression profile data from the GSE9348 and GSE20916 datasets in GEO.

Clinical Tissue Sample Collection

Between March and December of 2020, we collected tumor tissues and adjacent normal tissues from twelve patients with COAD at Jiangsu Provincial Hospital of Integrated Traditional Chinese and Western Medicine. The adjacent normal

Table 1 Association Between NAT1 Expression and Clinicopathological Parameters in Patients with COAD

Characteristic	Low expression of NAT1	High expression of NAT1	P value
n	239	239	
T stage, n (%)			0.010
T1	3 (0.6%)	8 (1.7%)	
T2	31 (6.5%)	52 (10.9%)	
T3	168 (35.2%)	155 (32.5%)	
T4	37 (7.8%)	23 (4.8%)	
N stage, n (%)			<0.001
N0	110 (23%)	174 (36.4%)	
N1	66 (13.8%)	42 (8.8%)	
N2	63 (13.2%)	23 (4.8%)	
M stage, n (%)			0.003
M0	164 (39.5%)	185 (44.6%)	
M1	45 (10.8%)	21 (5.1%)	
Pathologic stage, n (%)			<0.001
Stage I	29 (6.2%)	52 (11.1%)	
Stage II	73 (15.6%)	114 (24.4%)	
Stage III	88 (18.8%)	45 (9.6%)	
Stage IV	45 (9.6%)	21 (4.5%)	
Primary therapy outcome, n (%)			0.045
PD	16 (6.4%)	9 (3.6%)	
SD	3 (1.2%)	1 (0.4%)	
PR	8 (3.2%)	5 (2%)	
CR	86 (34.4%)	122 (48.8%)	
Gender, n (%)			0.783
Female	115 (24.1%)	111 (23.2%)	
Male	124 (25.9%)	128 (26.8%)	
Age, n (%)			0.162
<=65	105 (22%)	89 (18.6%)	
>65	134 (28%)	150 (31.4%)	
Weight, n (%)			0.911
<=90	105 (38.5%)	84 (30.8%)	
>90	48 (17.6%)	36 (13.2%)	
Height, n (%)			0.789
<170	73 (28.5%)	54 (21.1%)	
≥170	71 (27.7%)	58 (22.7%)	
BMI, n (%)			0.517
<25	46 (18%)	41 (16%)	
≥25	98 (38.3%)	71 (27.7%)	
Residual tumor, n (%)			0.733
R0	169 (45.2%)	177 (47.3%)	
R1	2 (0.5%)	2 (0.5%)	
R2	14 (3.7%)	10 (2.7%)	
CEA level, n (%)			0.006
<=5	87 (28.7%)	109 (36%)	
>5	66 (21.8%)	41 (13.5%)	
Perineural invasion, n (%)			0.115
NO	65 (35.9%)	70 (38.7%)	
YES	29 (16%)	17 (9.4%)	

(*Continued*)

Table 1 (Continued).

Characteristic	Low expression of NAT1	High expression of NAT1	P value
Lymphatic invasion, n (%)			<0.001
NO	117 (27%)	149 (34.3%)	
YES	102 (23.5%)	66 (15.2%)	
Colon polyps present, n (%)			0.775
NO	92 (36.9%)	70 (28.1%)	
YES	47 (18.9%)	40 (16.1%)	
Age, median (IQR)	68 (55, 76)	69 (61, 78)	0.057

tissue samples were obtained from a distance of more than three cm from the tumor margin and were subjected to microscopic evaluation to ensure the absence of abnormally proliferating cells. All subjects were confirmed to have COAD through pathological diagnosis and had undergone radical surgical resection. None of the patients had any other malignant tumors. The study received approval from the Medical Ethics Committee of Jiangsu Provincial Hospital of Integrated Traditional Chinese and Western Medicine, and all patients provided written informed consent.

Western Blot Assay (WB)

Proteintech company provided the antibodies for the Western blot assay, namely mouse anti-human NAT1 (67,942-1-Ig) and rabbit anti-human GAPDH (10,494-1-AP). Tissue protein extraction was initially performed using RIPA lysis buffer, and protein concentration was subsequently determined. Electrophoresis on a 10% SDS-polyacrylamide gel was then utilized to separate the proteins, which were subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane. Following this, the PVDF membrane underwent a two-hour blocking process in Tris-buffered saline (TBST) containing 5% skim milk at ambient temperature. Subsequently, the membrane was subjected to an overnight incubation period at 4°C with the aforementioned antibodies-mouse anti-human NAT1 and rabbit anti-human GAPDH. This was followed by a one-hour incubation phase with secondary antibodies at room temperature. Finally, the presence of the protein was identified using an enhanced chemiluminescence detection system, which served as the conclusive verification step.

Quantitative Real-Time Reverse-Transcription PCR Assay (RT-qPCR)

The COAD tissue samples were subjected to RNA extraction, followed by assessment of purity, concentration, and integrity. The TRIzol reagent (ThermoFisher, CA, USA) was employed for the extraction process. The resulting RNA was subjected to reverse transcription using the PrimeScript™ RT Reagent Kit (Takara, Dalian, China), leading to the construction of cDNA libraries and selection of specific target genes. RT-qPCR was performed using SYBR Premix Ex Taq™ (Takara Shuzo, Japan) on a real-time PCR system (Roche, Meylan, France). The comparative $2^{-\Delta\Delta CT}$ method was utilized to determine the relative expression of NAT1 mRNA. Each sample underwent three replicate experiments. The forward primer for NAT1 was 5'-GGTG ACCATCAGTGACAGGAAG-3', and the reverse primer was 5'-CTGTCAAAAGGAAGATGGCAGGC-3'. The forward primer for GAPDH was 5'-GTCTCCTCTGACTTCAACAGCG-3', and the reverse primer was 5'-ACCACCCTGTT GCTGTAGCCAA-3'.

PrognoScan Database Analysis

PrognoScan is a comprehensive online database designed to assist researchers in evaluating the association between gene expression and survival outcomes across a variety of tumor types.[18] It achieves this by aggregating, integrating, and analyzing an extensive range of publicly available gene expression datasets, along with their corresponding clinical information, to assess the impact of gene expression on patient prognosis. In this study, we utilized the PrognoScan database to analyze the correlation between NAT1 expression and prognosis in colon cancer, including overall survival (OS), disease-specific survival (DSS), and progression-free interval (PFI).

Gene Set Enrichment Analysis

The GSEA is a statistical methodology utilized for the interpretation of gene expression data. It can systematically identifies groups of genes that exhibit variation within the given dataset and are relevant to biological processes, pathways, or functional categories.[19] Gene expression data were divided into high-expression and low-expression groups based on the median expression level of NAT1 in the TCGA-COAD dataset. The GSEA software was employed to perform pathway enrichment analysis on NAT1, utilizing the c2.cp.kegg.v7.2.symbols.gmt dataset from the Molecular Signature Database (MsigDB) as functional gene sets.[20] Enrichments were considered significant if they met the following criteria: adjusted p-value <0.05 and false discovery rate (FDR) <0.25.

Immune Infiltration Analysis

Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT) is a computational method based on gene expression profiling. It can identify and quantify various cell types in complex mixed tissue samples.[21] CIBERSORT provides researchers the capacity to analyze heterogeneous biological samples, such as tumor tissues, and determine the relative abundance of various immune cell subgroups and other cell types within them. In our study, we employed the CIBERSORT algorithm to analyze the immune infiltration of 22 different immune cell types in 478 cases of colon cancer. Subsequently, we compared the significant differences in each type of immune cell between the NAT1 low-expression group and high-expression group.

Statistical Analysis

The R programming language was utilized to conduct statistical analyses.[22] For quantitative data conforming to a normal distribution, the mean and standard deviation were expressed as $\bar{x} \pm s$. Comparisons between two groups were conducted using an independent samples *t*-test, while comparisons among multiple groups were performed using a one-way analysis of variance (ANOVA). Pairwise comparisons were subsequently conducted using the least significant difference *t*-test (LSD-t). Survival analyses were conducted using the Kaplan-Meier method, complemented with a Log rank test. Statistical significance was established when the p-value was less than 0.05.

Results

Expression Levels of NAT1 in COAD

To investigate the expression level of NAT1 across various cancer types, we utilized the TIMER database for analysis. Our results revealed that NAT1 exhibited considerable differential expression in the majority of cancer types. Particularly, in COAD samples, the mRNA expression level of NAT1 was significantly reduced in comparison to normal colon tissues (Figure 1A).

To further elucidate the differential expression of NAT1 between COAD tissues and normal colon tissues, we utilized RNAseq data and associated clinical information from the TCGA database, consisting of 478 COAD samples and 41 normal colon tissue samples. Our analysis demonstrated a statistically significant decrease in NAT1 expression in COAD tissues (P<0.001, as shown in Figure 1B). Subsequently, we analyzed 41 paired COAD samples and their adjacent normal tissues, and our findings corroborated that NAT1 expression is indeed lower in COAD tissues (P<0.001, Figure 1C). To validate these findings, we downloaded two COAD datasets from the GEO database: GSE20916 and GSE9348. Results from both datasets independently confirmed that, relative to normal colon tissues, NAT1 is significantly down-regulated in COAD tissues (P<0.001, Figure 1D and E).

Correlation Between NAT1 and Clinicopathological Features

We utilized COAD RNA-seq data from the TCGA database to investigate the relationship between NAT1 expression and clinicopathological features. COAD patients were divided into two cohorts, a high-expression group (N=239) and a low-expression group (N=239), using the expression median value of NAT1 as the dividing metric. The results showed that the expression of NAT1 was correlated with T stage (P=0.01), N stage (P<0.001), M stage (P=0.003), pathological stage (P<0.001), Primary therapy outcome (P=0.045), CEA level (P=0.006) and Lymphatic invasion

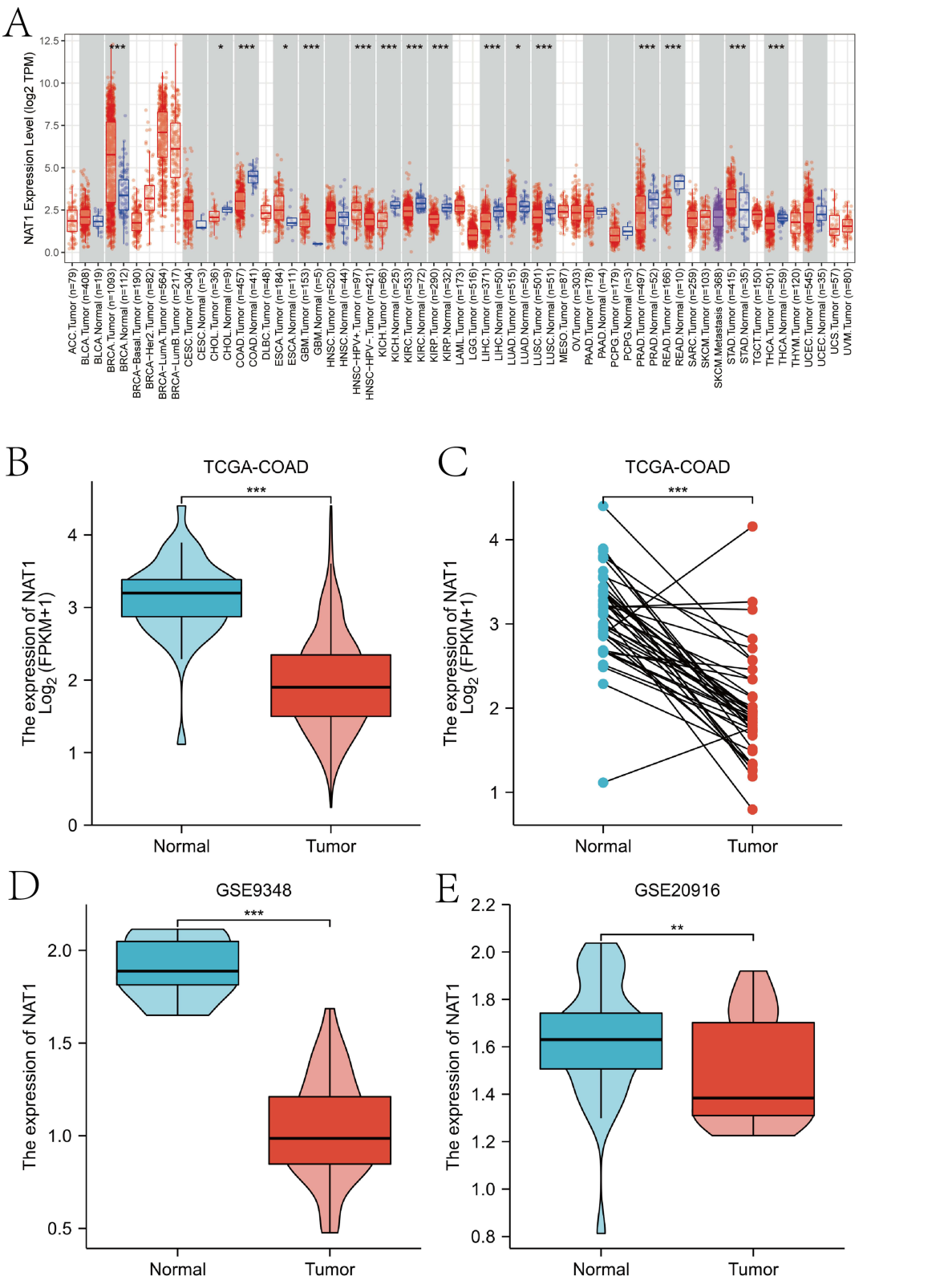

Figure 1 Expression levels of NAT1 mRNA in COAD from TIMER, TCGA and GEO databases. (**A**) Expression levels of NAT1 in different cancer types from TIMER database. (**B**) Expression of NAT1 mRNA was significantly down-regulated in COAD tumor tissues compared to normal colon tissues from the TCGA-COAD dataset. (**C**) Significantly decreased expression of NAT1 mRNA in COAD tumor tissues compared to adjacent paired normal colon tissues in the TCGA-COAD data. (**D**) GSE9348 dataset reveals a significantly lower NAT1 mRNA expression in COAD than in normal colon tissues. (**E**) GSE20916 dataset reveals a significantly lower NAT1 mRNA expression in COAD than in normal colon tissues.
Notes: *P<0.05, **P<0.01, ***P<0.001.

were significantly associated (P<0.001). Contrarily, the statistical significance (P>0.05) of the correlation between NAT1 expression and demographic and clinical characteristics such as gender, age, weight, height, BMI, residual tumor, perineural invasion, and presence of colon polyps was not achieved. Further analyses (Figure 2A–D) revealed that low expression of NAT1 is significantly correlated with T stage (T1/T2 vs T3/T4, P<0.001, Figure 2A), N stage (N0 vs N1/N2, P<0.001, Figure 2B), M stage (M0 vs M1, P<0.01, Figure 2C), and pathological stage (Stage I/II vs Stage III/IV, P<0.001, Figure 2D). Additionally, univariate logistic regression analyses (Table 2) substantiated that low expression of NAT1 was significantly associated with T stage (OR=0.492, 95% CI: 0.306 to 0.78, P=0.003), N stage (OR=0.319, 95% CI: 0.217 to 0.465, P<0.001), M stage (OR=0.414, 95% CI: 0.233 to 0.715, P=0.002), pathological stage (OR=0.714, 95% CI: 0.519 to 0.980, P=0.038), CEA level (OR=2.017, 95% CI: 1.251 to 3.279, P=0.004), and lymphatic invasion (OR=0.508, 95% CI: 0.342 to 0.751, P<0.001).

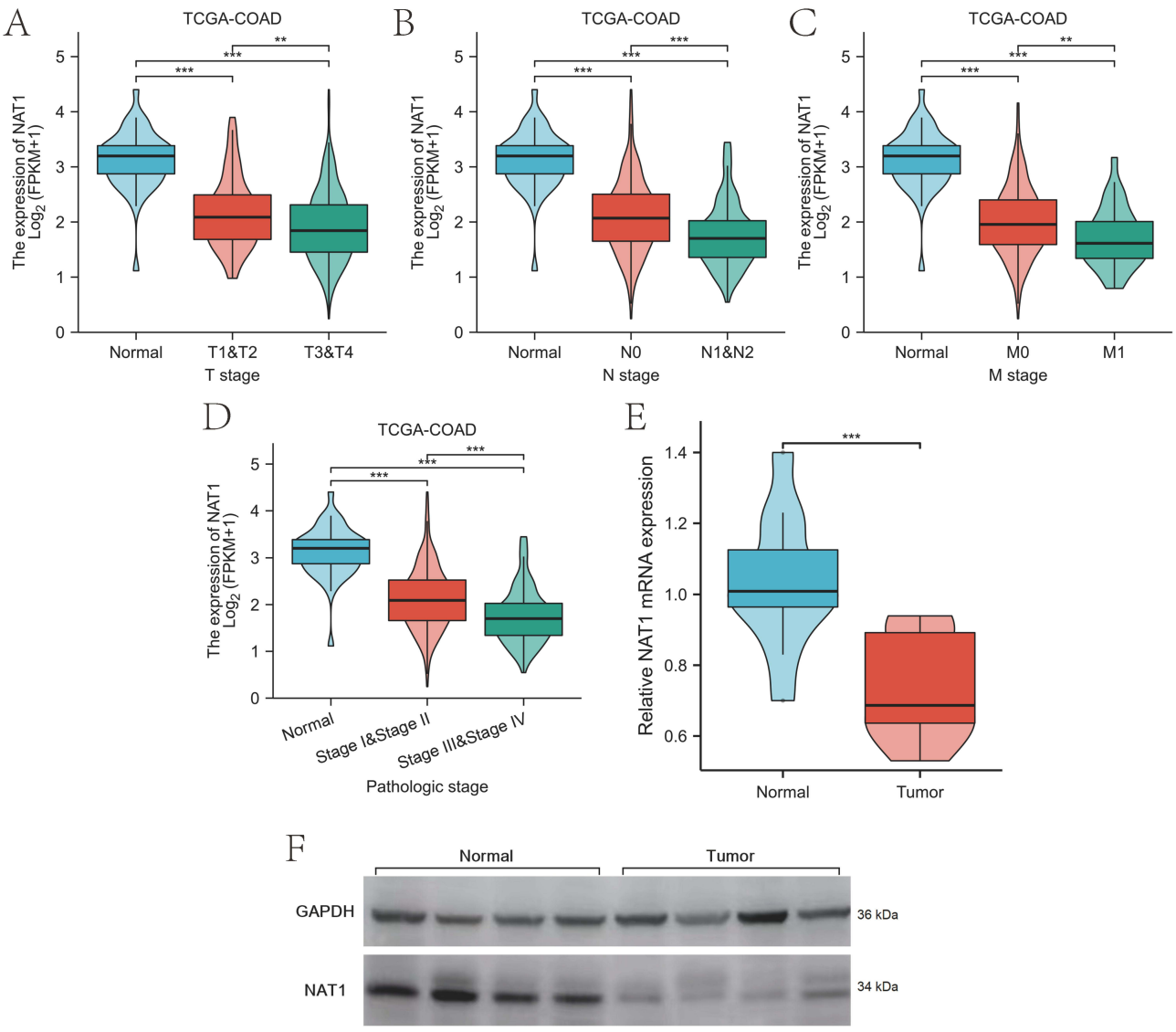

Figure 2 Expression levels of NAT1 in COAD clinical tissues. (**A-D**) Expression levels of NAT1 mRNA are analyzed using the TCGA-COAD dataset based on T, N, M, and pathological stages. (**E**) RT-qPCR analysis is conducted on COAD tissues and adjacent paired normal colon tissues. (**F**) Western blot (WB) analysis is conducted on COAD tissues and adjacent paired normal colon tissues.

Notes: **P<0.01; ***P<0.001.

Abbreviations: RT-qPCR, Quantitative reverse transcription polymerase chain reaction.

Table 2 Logistic Regression Analysis of the Relationship Between NAT1 Expression and Clinicopathological Parameters in COAD

Characteristics	Total(N)	Odds Ratio(OR)	P value
T stage (T3&T4 vs T1&T2)	477	0.492 (0.306–0.780)	0.003
N stage (N1&N2 vs N0)	478	0.319 (0.217–0.465)	<0.001
M stage (M1 vs M0)	415	0.414 (0.233–0.715)	0.002
Pathologic stage (Stage III&Stage IV vs Stage I&Stage II)	467	0.305 (0.207–0.446)	<0.001
Gender (Male vs Female)	478	1.069 (0.747–1.532)	0.714
Age (<=65 vs >65)	478	0.757 (0.524–1.091)	0.136
Weight (<=90 vs >90)	273	1.067 (0.636–1.799)	0.807
Height (<170 vs ≥170)	256	0.906 (0.552–1.484)	0.694
BMI (<25 vs ≥25)	256	1.230 (0.730–2.071)	0.435
Residual tumor (R1&R2 vs R0)	374	0.716 (0.322–1.551)	0.400
CEA level (<=5 vs >5)	303	2.017 (1.251–3.279)	0.004
Perineural invasion (YES vs NO)	181	0.544 (0.270–1.073)	0.083
Lymphatic invasion (YES vs NO)	434	0.508 (0.342–0.751)	<0.001
History of colon polyps (YES vs NO)	408	1.325 (0.883–1.993)	0.175

Detection of NAT1 Expression in Clinical Samples

The expression levels of NAT1 in COAD tissues and their paired adjacent normal tissues, including mRNA and protein, were assessed through the utilization of RT-qPCR and WB experiments. The results, as depicted in Figures 2E and F, indicated a significant down-regulation of both mRNA and protein expression of NAT1 in COAD tissues in comparison to adjacent normal tissues (Figure S1).

Low Expression of NAT1 Predicts Poor Prognosis in COAD Patients

In the pursuit to elucidate the prognostic implications of NAT1 expression in COAD patients, we employed the GSE14333 and GSE17536 datasets from the PrognoScan database. We discovered a significant correlation between low expression of NAT1 and patient prognosis across both datasets. Specifically, within the GSE14333 dataset, low expression of NAT1 was a significant correlation with poorer DFS (HR=2.43, 95% CI: 1.32 to 4.47, Cox P=0.004, Figure 3A). This trend was replicated in the GSE17536 dataset, where diminished NAT1 expression was a significant correlation with poorer DFS (HR=2.76, 95% CI: 1.33 to 5.73, Cox P=0.007, Figure 3B), DSS (HR=2.21, 95% CI: 1.24 to 3.92, Cox P=0.007, Figure 3C), and OS (HR=1.69, 95% CI: 1.05 to 2.73, Cox P=0.031, Figure 3D). Taken together, these findings suggest that NAT1 expression may serve as a valuable prognostic indicator for COAD patients.

To further verify the reliability of the relationship between NAT1 expression level and prognosis, we analyzed the TCGA-COAD dataset. The results showed that low expression of NAT1 was significantly associated with poorer OS (HR=0.62, 95% CI: 0.42 to 0.92, P=0.017, Figure 3E), PFI (HR=0.62, 95% CI: 0.44 to 0.89, P=0.008, Figure 3F) and DSS (HR=0.58, 95% CI: 0.35 to 0.97, P=0.036, Figure 3G). In addition, we performed univariate and multivariate Cox regression analyses to evaluate factors affecting the prognosis of COAD patients. The results of the univariate analysis showed that factors such as T stage, N stage, M stage, pathological stage, age, NAT1 expression level, and residual tumor all affected the prognosis of COAD patients (P<0.05). The results of multivariate Cox regression analysis further pointed out that N stage, pathological stage, age, and residual tumor were all independent risk factors for poor survival in COAD patients (Table 3).

NAT1 is a Potential Diagnostic Biomarker for COAD

We utilized receiver operating characteristic (ROC) to investigate the capacity of NAT1 expression levels to distinguish between COAD and normal tissues. First, we evaluated the GSE20916 dataset, which revealed an area under the curve (AUC) for NAT1 of 0.713 (95% CI: 0.578 to 0.848; Figure 3H). To further corroborate these findings, we analyzed the TCGA-COAD dataset, which unveiled an AUC value for NAT1 of 0.912 (95% CI: 0.864 to 0.959; Figure 3I), indicating

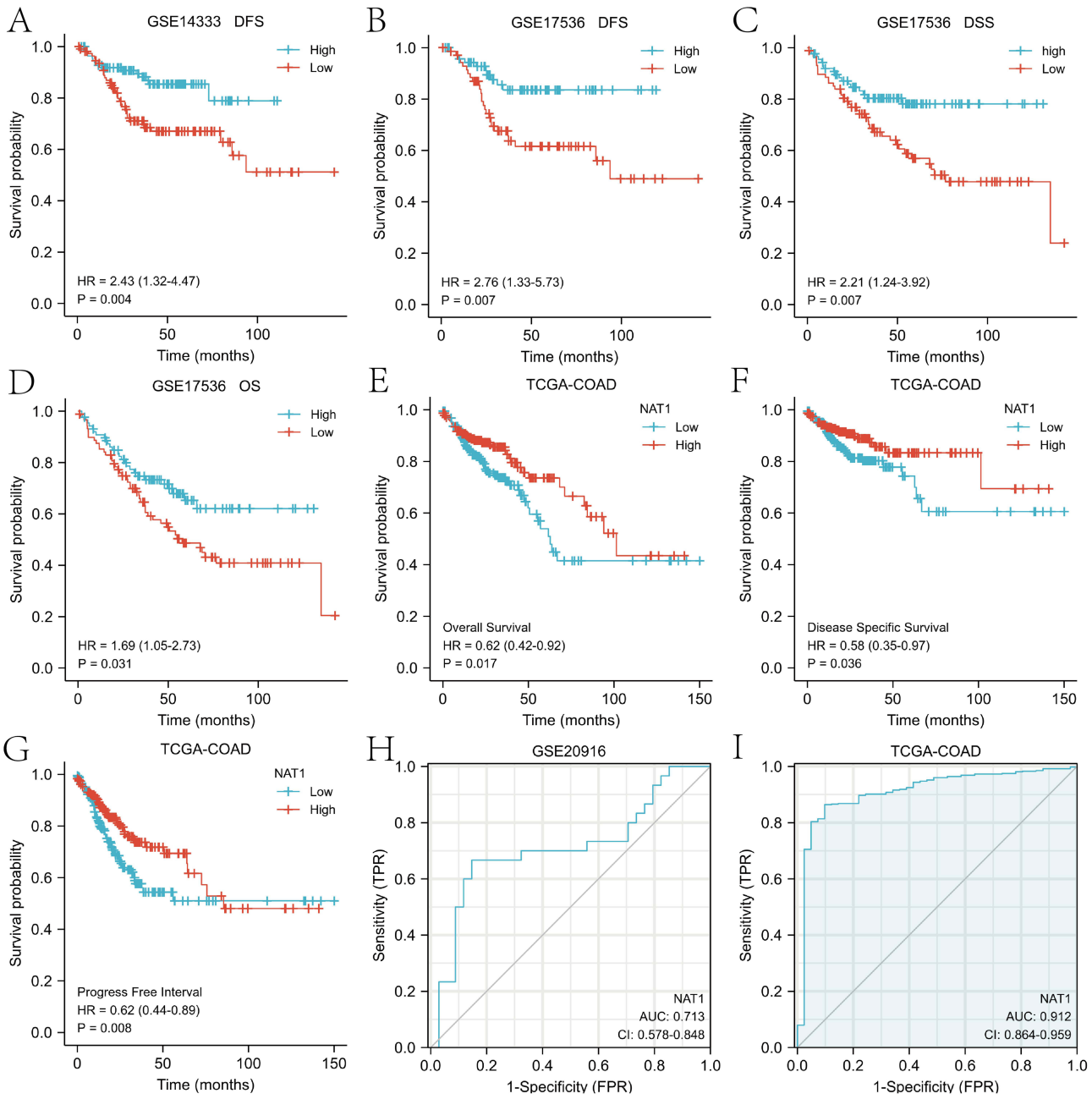

Figure 3 Diagnostic and prognostic value of NAT1 in COAD. (**A**) Relationship between NAT1 expression and DFS in the GSE14333 dataset. (**B-D**) Relationship between NAT1 expression and DFS, DSS and OS in the GSE17536 dataset. (**E-G**) Association of low or high NAT1 expression with OS, DSS and PFI in patients with COAD in the TCGA-COAD dataset. (**H-I**) ROC analysis of NAT1 in COAD, with an AUC of 0.713 (95% CI = 0.578 to 0.848) for the GSE20916 dataset and 0.912 (95% CI = 0.864 to 0.959) for the TCGA-COAD dataset.

Abbreviations: OS, overall survival; DFS, disease free survival; DSS, disease specific survival; PFI, progress free interval; ROC, Receiver operating characteristic; AUC, area under the curve.

that NAT1 had high accuracy in distinguishing COAD from normal tissue. Combining the results of the analysis of the two datasets, NAT1 could serve as a good diagnostic biomarker for COAD patients.

Biological Functions and Pathways of NAT1 in COAD

To explore the possible biological functions and related pathways of NAT1, we selected the genes co-expressed with NAT1, and used the clusterProfiler software to perform Gene Ontology (GO) enrichment analysis.[23] GO analysis consists of three parts: biological process (BP), cellular component (CC), and molecular function (MF). In the GO term analysis

Table 3 Univariate and Multivariate Cox Regression Analyses of Clinicopathological Factors Associated with OS in Patients with COAD

Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
T stage	476				
T1&T2	94	Reference			
T3&T4	382	3.072 (1.423–6.631)	0.004	7.412 (0.997–55.111)	0.050
N stage	477				
N0	283	Reference			
N1&N2	194	2.592 (1.743–3.855)	<0.001	0.275 (0.099–0.760)	0.013
M stage	414				
M0	348	Reference			
M1	66	4.193 (2.683–6.554)	<0.001	1.774 (0.893–3.525)	0.102
Pathologic stage	466				
Stage I&Stage II	267	Reference			
Stage III&Stage IV	199	2.947 (1.942–4.471)	<0.001	7.176 (2.079–24.775)	0.002
Gender	477				
Female	226	Reference			
Male	251	1.101 (0.746–1.625)	0.627		
Age	477				
>65	283	Reference			
<=65	194	0.621 (0.406–0.951)	0.028	0.510 (0.283–0.919)	0.025
Residual tumor	373				
R0	345	Reference			
R1&R2	28	4.364 (2.401–7.930)	<0.001	1.792 (0.874–3.674)	0.111
NAT1	477				
Low	238	Reference			
High	239	0.618 (0.417–0.917)	0.017	0.689 (0.395–1.200)	0.188

of BP, we found that processes such as membrane potential regulation, postsynaptic membrane potential regulation, keratinization, synapse assembly, and DNA replication-dependent nucleosome assembly were significantly enriched (Figure 4A). The GO term analysis of CC further pointed out that the genes co-expressed by NAT1 were mainly enriched in synaptic membranes, synaptic membrane intrinsic components, postsynaptic membranes, synaptic membrane components, and nucleosomes (Figure 4B). Finally, MF analysis revealed that NAT1 may be associated with significant enhancement of substrate-specific channel activity, channel activity, passive transmembrane transport activity, ion channel activity, bitter taste receptor activity, and ion-gated channel activity (Figure 4C). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identified significantly enriched pathways, including neuroactive ligand-receptor interactions, taste transduction, nicotine addiction, bile secretion, and retinol metabolism (Figure 4D).

To further investigate the function and biological impact of NAT1, we performed a GSEA analysis on NAT1. The results showed that multiple signaling pathways were significantly enriched when NAT1 expression was low, including neuroactive ligand-receptor interaction, olfactory transduction, responder olfactory signaling pathway, responder base excision repair, and ECM receptor interaction (Figure 5A–E, Table 4). However, other pathways such as the D4GDI pathway, MHC pathway, CTL pathway, and IL17 pathway showed significant enrichment in the presence of high expression of NAT1 (Figure 5F–I).

Correlation Between NAT1 Expression and Immune Cell Infiltration Level in COAD

Tumor-infiltrating lymphocytes (TILs), including T cells, B cells, and natural killer (NK) cells, are a type of lymphocytes present in the tumor microenvironment. TILs play key roles in tumor initiation, progression, prognosis, and immunotherapy. Because of this, we further investigated the association between NAT1 gene expression and immune cell

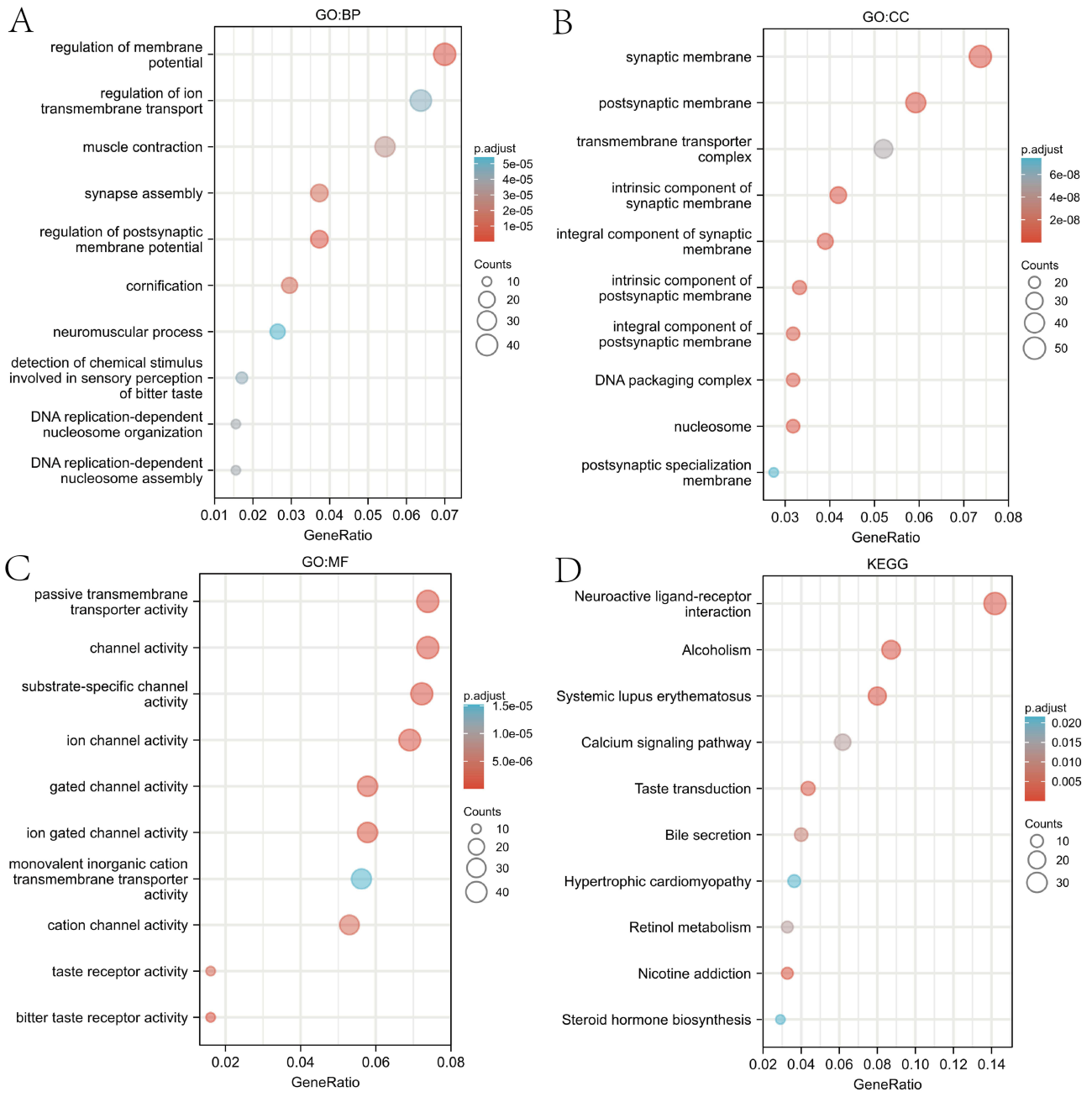

Figure 4 GO and KEGG enrichment analysis of NAT1 gene. (**A-C**) GO enrichment analysis showing BP (biological process), CC (cellular component) and MF (molecular function) of genes co-expressed with NAT1. (**D**) KEGG enrichment analysis of genes co-expressed with NAT1.

infiltration in COAD (Figure 6A). Our analysis results showed that the expression of NAT1 was positively correlated with the infiltration level of various immune cells, including Th17 cells (R=0.22, P<0.001, Figure 6B), B cells (R=0.223, P<0.001, Figure 6C), T helper cells (R=0.244, P<0.001, Figure 6D), T cells (R=0.242, P<0.001, Figure 6E), Th2 cells (R=0.409, P<0.001, Figure 6F), cells Toxic cells (R=0.138, P<0.001), eosinophils (R=0.12, P<0.001), Tgd cells (R=0.118, P=0.007), aDC (R=0.148, P<0.001) and CD8 T cells (R=0.148, P<0.001). However, the expression of the NAT1 was correlated with the infiltration of NK cells (R=−0.208, P<0.001, Figure 6G), pDC cells (R=−0.11, P=0.02), and Tem cells (R=−0.136, P<0.001) levels are negatively correlated. These findings imply that NAT1 may play a role in regulating immune cell infiltration in COAD.

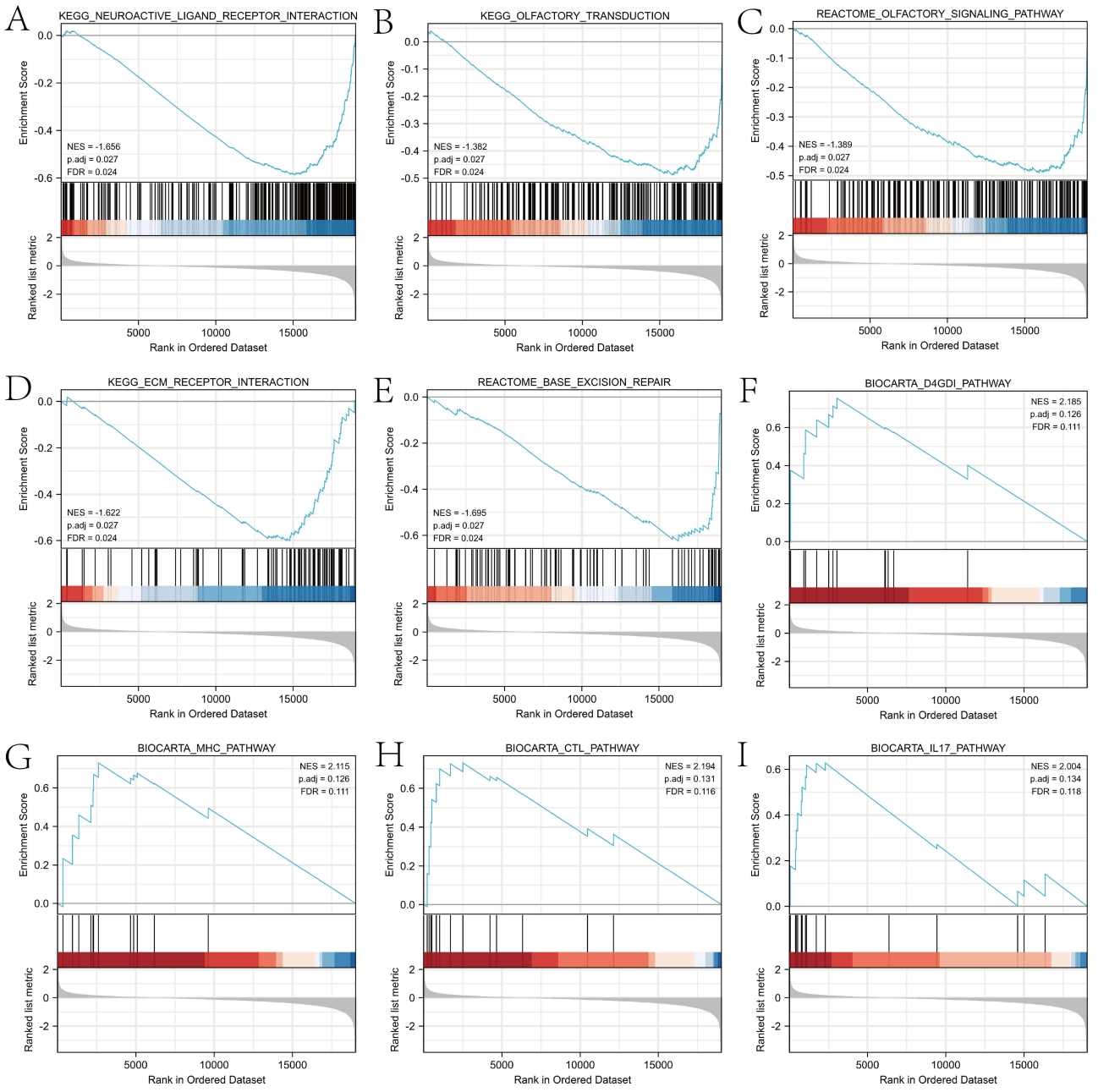

Figure 5 Enrichment plots from the Gene Set Enrichment Analysis (GSEA). NAT1 were significantly enriched several pathways including: (**A**) Neuroactive ligand-receptor Interaction, (**B**) Olfactory transduction, (**C**) Olfactory signaling pathway, (**D**) ECM receptor interaction, (**E**) Base excision repair, (**F**) D4GDI pathway, (**G**) MHC pathway, (**H**) CTL pathway, and (**I**) IL17 pathway.

Abbreviations: NES, Normalized Enrichment Scores; FDR, False Discovery Rate.

Discussion

Colon cancer is one of the most prevalent malignancies in the gastrointestinal tract. The lack of specific clinical symptoms in its early stages often results in most patients presenting with advanced disease at the time of diagnosis, leading to a higher mortality rate.[24] Consequently, improving early detection and diagnostic capabilities for colorectal cancer is of utmost importance. Colonoscopy combined with pathological biopsy is deemed the "gold standard" for colorectal cancer diagnosis. However, the painful examination process leads to low patient compliance.[25] As auxiliary diagnostic tools, serum tumor markers, such as carcinoembryonic antigen (CEA), carbohydrate antigen 19–9 (CA19-9), and carbohydrate antigen 72–4 (CA72-4), are widely used. Nonetheless, these markers' relatively low sensitivity and specificity constrain their effectiveness in early detection and diagnosis and hinder their widespread application in clinical practice.[26] Therefore, it is of excellent research significance to find

Table 4 Results of Gene Set Enrichment Analysis (GSEA) for NAT1

Description	setSize	enrichmentScore	pvalue	p.adjust	qvalue
KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	270	−0.5872	0.0009	0.027	0.0239
KEGG_OLFACTORY_TRANSDUCTION	370	−0.4878	0.0009	0.027	0.0239
REACTOME_OLFACTORY_SIGNALING_PATHWAY	376	−0.4903	0.0009	0.027	0.0239
REACTOME_BASE_EXCISION_REPAIR	90	−0.6251	0.001	0.0271	0.0239
KEGG_ECM_RECEPTOR_INTERACTION	83	−0.6015	0.001	0.0271	0.0239
BIOCARTA_D4GDI_PATHWAY	12	0.7536	0.0064	0.1257	0.1112
BIOCARTA_MHC_PATHWAY	12	0.7293	0.0064	0.1257	0.1112
BIOCARTA_CTL_PATHWAY	13	0.7304	0.0068	0.131	0.1159
BIOCARTA_IL17_PATHWAY	15	0.6281	0.0074	0.1335	0.1181

biomarkers and therapeutic targets with higher sensitivity and specificity to improve the ability of early diagnosis and prognosis of colon cancer.

Aberrant expression and dysfunction of the NAT family have been firmly associated with the pathogenesis and progression of many diseases, encompassing malignancies, autoimmune disorders, and neurodegenerative diseases.[9,10] NAT1, a member of the NAT family, executes critical roles in crucial biological processes such as drug metabolism and biosynthesis. Existing research has identified a close relationship between NAT1 and various cancer types, whereby its differential expression and polymorphisms could potentially influence the onset of numerous malignancies, including breast cancer, bladder cancer, and prostate cancer.[11–13] Despite the extensive exploration of NAT1's function across numerous cancer types, its prognostic implications and biological function within COAD remain elusive. Therefore, this study aimed to reveal the prognostic value of NAT1 in COAD and to further explore its biological function in COAD by comprehensively analyzing public datasets as well as molecular experiments on clinical samples.

In this study, we first revealed the differential expression of NAT1 mRNA in different cancer types. Based on the data of the TCGA and GEO database, combined with the experiments of clinical samples, we further conducted an in-depth study on the expression of NAT1 mRNA in COAD. The results clearly showed that NAT1 mRNA and protein levels were significantly reduced in COAD tissues compared with normal colon tissues. It is especially noteworthy that the expression level of NAT1 was significantly correlated with the clinicopathological features of COAD patients, implying that the reduction of NAT1 expression may be related to the severity of COAD. In addition, we also found that low expression of NAT1 was a significant correlation with decreased OS, DSS, DFS, and PFI in COAD patients. Cox regression analysis reveals NAT1 as an independent prognostic factor for COAD patients. The ROC analysis confirmed the high accuracy of NAT1 in diagnosing COAD patients. These discoveries underscore the key role of NAT1 in the pathogenesis of COAD, and provide new directions for future research.

To gain insight into the potential role of NAT1 in COAD, we performed GO and KEGG analysis of NAT1 co-expressed genes using TCGA data. In GO analysis, we found that biological processes closely related to cell signaling functions were significantly enriched in BP, including membrane potential regulation, postsynaptic membrane potential regulation, cornification, synapse assembly, and DNA replication-dependent Nucleosome assembly, etc. In the Cellular Component (CC) category, elements such as synaptic membranes, intrinsic constituents of synaptic membranes, post-synaptic membranes, components of synaptic membranes, and nucleosomes displayed pronounced enrichment. MF analysis revealed that substrate-specific channel activity, channel activity, passive transmembrane transport activity, ion channel activity, bitter taste receptor activity, and ion-gated channel activity were significantly enrichment. KEGG pathway analysis revealed that neuroactive ligand-receptor interaction, taste transduction, nicotine addiction, bile secretion, and retinol metabolism were identified as significantly enriched pathways. Notably, the pathway of neuroactive ligand-receptor interaction has been proven to be involved in the onset and progression of various types of cancer, providing key clues about the possible role of NAT1 in COAD. Taken together, these results provide important insights for further investigating the potential role of NAT1 in COAD.

Finally, we explored the correlation between NAT1 and different immune cell populations. Our results revealed a significant correlation between the expression of NAT1 and the infiltration levels of various immune cells. The

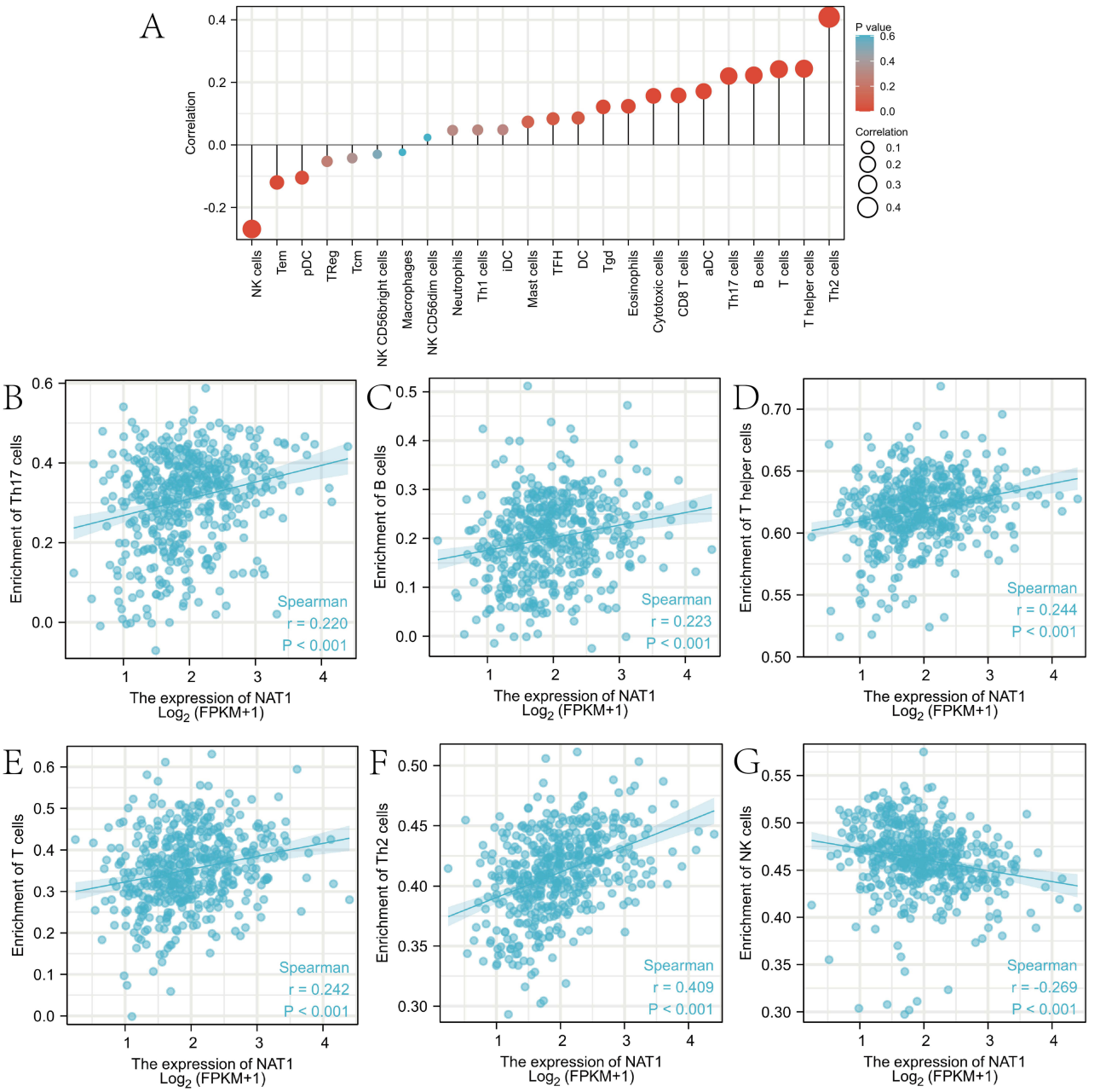

Figure 6 Association analysis of NAT1 gene expression with immune infiltration. (**A**) Association between NAT1 expression and 24 tumor-infiltrating lymphocytes. (**B-G**) Correlation of NAT1 expression with immune infiltration levels of (**B**) Th17 cells, (**C**) B cells, (**D**) T helper cells, (**E**) T cells, (**F**) Th2 cells, and (**G**) NK cells.

expression of NAT1 was positively correlated with the infiltration levels of aDC, B cells, CD8 T cells, cytotoxic cells, eosinophils, T cells, T helper cells, Tgd cells, Th17 cells, and Th2 cells, whereas it was correlated with NK cells, pDC cells and The level of infiltration of Tem cells was negatively correlated. It's worth highlighting that these immune cells play a key role in tumor initiation and progression, such as T cells, B cells, and natural killer (NK) cells. Moreover, the immune microenvironment of tumors and associated immune mechanisms significantly influence the progression of tumors and the efficacy of cancer treatments, bearing a close relation with clinical outcomes. Consequently, our results imply that NAT1 may play a role in regulating immune cell infiltration in COAD, particularly lymphocyte infiltration.

The innovation of our study is that this is the first time that the connection between NAT1 and COAD has been systematically explored. Nonetheless, we must acknowledge certain limitations within our work. The first limitation is

that our study relies heavily on bioinformatics analysis. While these tools have provided us with a wealth of valuable information, future studies should incorporate a greater number of in vivo and in vitro experiments to validate our findings. These experimental validations will help to further consolidate our research results and gain a deeper understanding of the role of NAT1 in COAD. Second, the number of databases used in this study is relatively limited. Although our preliminary results are somewhat convincing, cross-validation in more diverse datasets is extremely necessary. Through in-depth analysis of multiple datasets, it can enhance the reliability of our findings and provide a more solid foundation for further research. In summary, although our study has certain limitations, it is only the first step in our understanding of the role of NAT1 in COAD. We look forward to more in-depth studies in the future to overcome these limitations and reveal more mechanistic details.

Conclusion

In this study, we reveal the essential role of NAT1 in COAD. We found that NAT1 expression was significantly decreased in COAD tissues compared to adjacent paracancerous tissues. More importantly, the reduced expression of NAT1 was closely related to the pathological stage and poor prognosis of COAD, and these prognostic indicators included OS, DSS, and PFI. ROC analysis confirmed that NAT1 could be effectively used as a biomarker to distinguish COAD from normal colon tissue. Our analysis results suggest that NAT1 may affect the occurrence and progression of COAD by participating in the regulation of a series of biological processes including membrane potential regulation, postsynaptic membrane potential regulation, keratinization, and immune cell infiltration. Overall, our study reveals the underlying mechanism of NAT1 in the development of COAD and provides a theoretical basis for its application in tumor diagnosis and treatment.

Data Sharing Statement

The datasets generated and analyzed during the current study are available in the TCGA repository (https://portal.gdc.cancer.gov/) and The Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Approval and Consent to Participate

This study was approved by the Ethics Committee of Jiangsu Hospital of Integrated Traditional Chinese and Western Medicine. All participants provided signed informed consent in accordance with the Declaration of Helsinki.

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We appreciate the support of all participators involved in this study. 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; giving final approval of the version to be published; agreeing 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 report no conflicts of interest in this work.

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