BIOCELL
2023 47(3): 607-618

Increased *MAD2L2* expression predicts poor clinical outcome in Colon Adenocarcinoma

Haotong SUN[1,2]; Heying WANG[1,3]; Xin LI[1,2]; Yanjie HAO[1,2]; Jun LING[1,2]; Huan WANG[1,2]; Feimiao WANG[1,2]; Fang XU[1,2,*]

[1] School of Basic Medicine, Medical Genetics and Cell Biology, Ningxia Medical University, Yinchuan, 750004, China
[2] Key Laboratory of Reproduction and Genetics, Ningxia Medical University, Yinchuan, 750004, China
[3] Department of Human Anatomy and Histology Embryology, Ningxia Medical University, Yinchuan, 750004, China

Key words: *MAD2L2*, COAD, Poor prognosis, Immune infiltration, TCGA

Abstract: Background: Colon adenocarcinoma (COAD) is the second leading cause of cancer death worldwide thus, identification of COAD biomarkers is critical. Mitotic Arrest Deficient 2 Like 2 (*MAD2L2*) is a key factor in mammalian DNA damage repair and is highly expressed in many malignant tumors. This is a comprehensive study of *MAD2L2* expression, its diagnostic value, prognostic analysis, potential biological function, and impact on the immune system of patients with COAD. **Methods:** Gene expression, clinical relevance, prognostic analysis, diagnostic value, GO/KEGG cluster analysis, data obtained from TCGA, and bioinformatics statistical analysis were performed using the R package. Immune responses to *MAD2L2* expression in COAD were analyzed using TIMER. The expression of *MAD2L2* in HCT116 cells induced by the inflammatory factor TNF-α was detected using Western blot. **Results:** Our results underscore the clinical diagnostic value and potential biological significance of *MAD2L2* in patients with COAD. A high level of *MAD2L2* expression has been found in COAD and correlated with tumor status and colon polyps. ROC curve analysis showed that *MAD2L2* expression has high diagnostic value in COAD. Analysis of immune infiltration results showed that *MAD2L2* expression was positively correlated with neutrophil levels. The western blot results demonstrated that *MAD2L2* was dose-dependently present with TNF-α. GO/KEGG revealed that *MAD2L2* overexpressed and coexpressed genes were mostly involved in biological functions, including hypoxia response, response to reduced oxygen levels, mitochondrial translation elongation, and other processes. **Conclusion:** *MAD2L2* as a new COAD biomarker contributes to our understanding of how alterations in gene expression and the immunological environment contribute to the development of colon cancer. Following further investigation, *MAD2L2* may prove to be a viable target factor for clinical diagnosis and therapy of COAD.

Introduction

Colon adenocarcinoma (COAD) is the second leading cause of cancer-related death worldwide, with approximately 1.2 million new cases each year (Malayaperumal *et al.*, 2021). Despite significant advances in multimodal treatment options such as surgery, chemotherapy, radiation, and immunotherapy, the 5-year survival rate for patients with advanced COAD is just 6.6%, and the recurrence rate is substantial (Dulskas *et al.*, 2020). Gene mutations and differences in individual drug responses are barriers to cancer treatment (Zhang *et al.*, 2018; Saito *et al.*, 2021). With the update of the 8th edition of the American Joint Committee on Cancer (AJCC) colorectal cancer (CRC) staging system, molecular tests such as MSI, KRAS, NRAS, and BRAF have been recommended based on high-level "evidence-based medicine" evidence, indicating that colorectal cancer has changed from traditional. Based on the "group" diagnosis and treatment, it has entered the precise "individualized" medical treatment (Weiser, 2018). Key genes involved in carcinogenesis are considered therapeutic targets in precision medicine. As an alternative to traditional colon cancer treatment, targeted therapy is suitable for early diagnosis of COAD (Yu *et al.*, 2020). Anti-vascular endothelial growth factors (anti-VEGF) receptor (Fang *et al.*, 2017), anti-epidermal growth factor receptor (anti-EGFR) (Hong *et al.*, 2020), PD-1 (Zhao *et al.*, 2020), CTLA-4 (Ben *et al.*, 2021), and other genes are now utilized to treat COAD. Although molecular targeted therapy has shown good clinical effects, biomarker screening for the prognosis of COAD is still in the preliminary stage, and does not meet

*Address correspondence to: Fang Xu, xufang@nxmu.edu.cn
Received: 06 September 2022; Accepted: 15 November 2022

Doi: 10.32604/biocell.2023.026445

clinical needs. New target molecules are required to effectively treat COAD patients. Consequently, clarifying the development of novel biomarkers as diagnostic and therapeutic targets for COAD is crucial. Mitotic Arrest Deficient 2 Like 2 (MAD2L2) is a structural subunit of mutagenic DNA polymerase and a major component of the shieldin complex. It is involved in a range of biological activities, including DNA damage repair, cell cycle progression, and apoptosis. MAD2L2, as a participant in the DNA damage repair process, is highly expressed in multiple malignant tumors (de Krijger *et al.*, 2021). MAD2L2 is highly expressed in epithelial ovarian cancer, and knockdown of MAD2L2 inhibits the proliferation of ovarian cancer cells, enhances chemosensitivity of ovarian cancer cells, and promotes apoptosis (Karakashev *et al.*, 2020). Knockdown of MAD2L2 leads to reduced colony formation and increased apoptosis in irradiated esophageal squamous cell carcinoma cells and a reduction in tumor weight after radiation in a xenograft nude mouse model (Gu *et al.*, 2019). In breast cancer cells, knockdown of MAD2L2 inhibits cell invasion and migration and promotes expression of transforming growth factor β1 (TGF-β1) (Feng *et al.*, 2016). These results imply that MAD2L2 may have a role in biological processes as well as act as a possible biomarker for the clinical diagnosis and prognostic prediction of COAD (Rimkus *et al.*, 2007). MAD2L2 overexpression was shown to be substantially related with reduced survival in a subset of human colorectal cancers. However, the present investigation did not assess the MAD2L2 gene expression's unique clinical diagnostic significance in individuals with COAD. We examined COAD gene expression in this research utilizing bioinformatics analysis and data from The Cancer Genome Atlas (TCGA) database. This demonstrates the feasibility of MAD2L2 as a clinical diagnostic marker molecule for COAD. Simultaneously, we used clinical factors and TCGA gene expression data to develop a predictive nomogram that would assist physicians in forecasting the chance of death and directing treatment choices for COAD patients.

Materials and Methods

Evidence from the TCGA database

Our data were derived from the RNA-seq data obtained from the COAD project of the TCGA database (https://portal.gdc.cancer.gov/). Gene expression, baseline data, clinical logistic correlation, prognostic analysis, clinical diagnostic value, and gene function clustering data were obtained through the R package. Our study excluded samples with insufficient "0" gene expression values and survival information. We retained the RNA-seq and clinical data for further study. A total of 478 COAD patients with comparable clinical symptoms were included in this investigation. Our study conforms with the publishing criteria established by TCGA.

Immune infiltrate analysis

Tumor Immune Estimation Resource (TIMER) (https://cistrome.shinyapps.io/timer/) was utilized to investigate the possibility of a link between MAD2L2 expression and tumor-infiltrating immune cells. We used a genetic module to determine the relationship of MAD2L2 in COAD with tumor-infiltrating immune cells, including CD4+ T cells, dendritic cells, B cells, neutrophils, and macrophages. TIMER developed a graph demonstrating the association between the degree of gene expression and the purity of the tumor. Furthermore, we investigated the connection between MAD2L2 and 24 immune-infiltrating cells in COAD. The markers for 24 immune cells were taken from an article in Immunity (Bindea *et al.*, 2013).

Cell culture and processing

The colon cancer cell line HCT116, used in the experiments, was obtained from the American Type Culture Center. Cells were cultured in DMEM (Vivacell; Shanghai, China) supplemented with 10% FBS (Vivacell; Shanghai, China) and penicillin-streptomycin (10,000 U/ml; Beyotime Institute of Biotechnology). Culture was performed at 37°C and 5% CO_2. When HCT116 cells reached 70% growth status, proteins were collected after treatment with TNF-α cytokines at different concentrations (0, 20, 20, 40, 60, 80, 100 ng/ml) for 24 h. TNF-α 50 mg (Peprotech, Suzhou, China).

Western blot

Western blotting was used to isolate and identify proteins. Total proteins were extracted using RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentration was then determined using the BCA kit (Nanjing Capgemini Biotechnology Co., Ltd., China). Proteins (10–20 μg/well) were passed through 10% SDS-PAGE at 80V, and then transferred onto 0.2 μm PVDF membranes under wet conditions at 200 mA. The blotted membranes were blocked with 5% skim milk for 1 h at room temperature. The antibody was diluted in primary antibody diluent. Primary antibodies were incubated at room temperature for 3 h. Secondary antibodies were incubated for 1 h at room temperature. Chemiluminescence signal was detected using Pierce™ ECL and analyzed by Image Lab software (version 5.2.1 Bio-Rad Laboratories, Inc., BIO-RAD, CA, USA). The following antibodies were used: primary antibody-TNF-α (cat. 8184s; 1:1,000; cell signaling, USA), MAD2L2 (cat. ab180579; 1:1,000; Abcam, UK), GAPDH (cat. AB-P-R001; 1:1,000; Hangzhou Xianzhi Biotechnology Ltd., China); and secondary antibody-goat anti-rabbit IgG H&L (HRP) (cat. ZB-2306; 1:10,000; Beijing Zhongshan Jinqiao, China).

Transwell

Transwell assay was used to detect cell invasion and migration. Cell migration was treated by taking cells in logarithmic growth phase and digesting the cells with trypsin. A 24-well plate was taken, 600 ul of complete medium was added to the lower chamber, and the transwell was placed in the plate and incubated for 48 h. Afterwards, 4% paraformaldehyde was added to fix the cells for 20 min, and then the cells were washed 3 times with PBS, stained with crystal violet for 15 min, and photographed and cell counted under a microscope (200X). The cell invasion was treated by first laying down the matrix gel. After that, a 24-well plate was taken, 600 ul of medium containing chemokine was added to the lower chamber, the transwell chambers after gelling were put into the well plate, 100–200 ul

of cell suspension was taken and mixed and added to the upper chamber, and the culture was fixed with 4% paraformaldehyde after 48 h, stained with crystalline violet for 15 min, and Matrigel and the non-migrated cells in the upper chamber were gently wiped with cotton swabs, PBS-washed, photographed and cell counted under a microscope (200X). The following materials were mainly used, transwell chambers (Corning Inc., USA), and Matrigel gel (BD Inc., USA).

Statistical analysis

All statistical analyses were performed using R package (version 3.6.3). To calculate MAD2L2 expression, the Mann–Whitney U test was used. The correlation between clinical features and MAD2L2 expression was analyzed using a binary logistic model. In calculating the 95% CI and HR, the Cox regression module to analyze univariate and multivariate models was used. To compare numerous clinical parameters and survival rates, a univariate survival analysis was performed on each individual patient. Using multivariate Cox analysis, we were able to determine the expression of MAD2L2 as well as the presence of additional pathological and clinical variables. The threshold for MAD2L2 expression was established at a *p*-value of less than 0.05.

Results

Baseline data characteristics

In February 2022, after screening, a total of 478 patients with clinical features related to MAD2L2 expression were obtained from the TCGA website. Table 1 lists the detailed clinical characteristics. Among the 478 participants, among those with low MAD2L2 expression, 121 were female (25.3%), and 118 were male (24.7%); among those with high expression of MAD2L2, 105 were female (22%), and 134 were male (28%). The age of all participants was cut off at 65 years. In terms of COAD pathological stage, among

TABLE 1

Clinical characteristics of the colon cancer patients

Characteristic	Low expression of *MAD2L2*	High expression of *MAD2L2*	*p*
n	239	239	
Gender, n (%)			0.169
Female	121 (25.3%)	105 (22%)	
Male	118 (24.7%)	134 (28%)	
Age, n (%)			0.226
<=65	104 (21.8%)	90 (18.8%)	
>65	135 (28.2%)	149 (31.2%)	
T stage, n (%)			0.028
T1	1 (0.2%)	10 (2.1%)	
T2	42 (8.8%)	41 (8.6%)	
T3	160 (33.5%)	163 (34.2%)	
T4	35 (7.3%)	25 (5.2%)	
N stage, n (%)			0.756
N0	138 (28.9%)	146 (30.5%)	
N1	56 (11.7%)	52 (10.9%)	
N2	45 (9.4%)	41 (8.6%)	
M stage, n (%)			0.276
M0	167 (40.2%)	182 (43.9%)	
M1	37 (8.9%)	29 (7%)	
Pathologic stage, n (%)			0.637
Stage I	39 (8.4%)	42 (9%)	
Stage II	89 (19.1%)	98 (21%)	
Stage III	69 (14.8%)	64 (13.7%)	
Stage IV	37 (7.9%)	29 (6.2%)	
CEA level, n (%)			0.966
<=5	99 (32.7%)	97 (32%)	
>5	53 (17.5%)	54 (17.8%)	
History of colon polyps, n (%)			<0.001
NO	149 (36.5%)	113 (27.7%)	
YES	53 (13%)	93 (22.8%)	

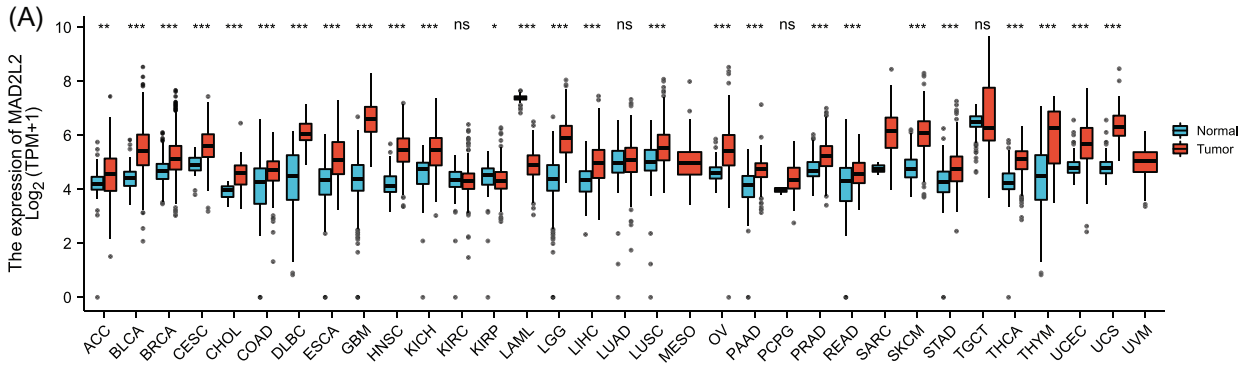

FIGURE 1. Expression level of *MAD2L2* gene in different tumors. (A) The expression status of the *MAD2L2* gene in different cancers or specific cancer subtypes was analyzed through UCSC XENA. ns, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

patients with low MAD2L2 expression, 39 patients were in Stage I (8.4%), 89 patients were in Stage II (19.1%), 69 patients were in Stage III (14.8%), and 37 patients were in Stage IV (7.9%); among patients with high MAD2L2 expression, 42 patients were in Stage I (9%), 98 patients were in Stage II (21%), 64 patients were in Stage III (13.7%), and 29 patients were in Stage IV (6.2%). History of colonic polyps, low expression of MAD2L2, 149 patients had no history of colonic polyps (36.5%) and 53 patients had a history of polyps (13%); among the highly expressed MAD2L2, 113 patients had no history of colon polyps (27.7%) and 93 patients had a history of colon polyps (22.8%). The median follow-up time among the 478 patients was 27.6 months.

MAD2L2 is expressed at a high level in COAD

MAD2L2 expression was increased in colon cancer, adrenocortical carcinoma, uroepithelial carcinoma of the bladder, invasive breast cancer, squamous and adenocarcinoma of the cervix, bile duct cancer, diffuse large B-cell lymphoma, esophageal cancer, glioblastoma multiforme, squamous cell carcinoma of the head and neck, low-grade (Fig. 1). RNA-seq research indicated that MAD2L2 expression was much greater in COAD cancer tissues than in paraneoplastic tissues in unpaired *vs.* paired samples (Figs. 2A and 2B). MAD2L2 mRNA expression was considerably elevated in COAD, as revealed by the findings.

High expression of MAD2L2 is associated with poor clinical prognosis

Table 2 summarizes the correlation between *MAD2L2* expression and clinical logistic characteristics in patients with COAD. Expression of *MAD2L2* was correlated with the history of colon polyps ($p < 0.001$) but not with other clinical features. Meanwhile, in the box plot of clinical features, we found that *MAD2L2* expression correlated with tumor status and history of colon polyps (Figs. 3A–3H). The results of a univariate study using Cox regression revealed that *MAD2L2* gene expression was a categorical dependent variable that was related with poor prognostic clinical characteristics (Table 3). The expression of *MAD2L2* was significantly correlated with age greater than 65 years ($p = 0.028$, [CI] = 1.052–2.463). N stage (N1: $p = 0.042$, [CI] = 1.019–2.771, N2: $p < 0.001$, [CI] = 2.593–6.329), M stage (M1: $p < 0.001$, [CI] = 2.683–6.554), pathological stage (Stage III: $p = 0.007$, [CI] = 1.436–9.448, Stage IV: $p < 0.001$, [CI] = 3.608–23.936), CEA level (greater than 5: $p < 0.001$, [CI] = 1.788–5.471) are relevant. These results suggest that high *MAD2L2* expression is highly correlated with poor clinical prognosis in COAD. We also examined the effect of high *MAD2L2* expression on the migration and invasion of COAD cells HCT116 and found that the migration and invasion expression of HCT116 cells were significantly increased when *MAD2L2* was highly expressed (Figs. 3I and 3J). This result indicates further validation that, high *MAD2L2* expression causes poor prognosis of COAD.

MAD2L2 as a predictor of COAD

The gene expression data for MAD2L2 was used to conduct a ROC curve analysis to determine the clinical diagnostic value of this gene. As shown in Fig. 4A, the AUC area of MAD2L2 was 0.93 when compared to paracancerous tissue *vs.* COAD. Using subgroup analysis, it was discovered that MAD2L2

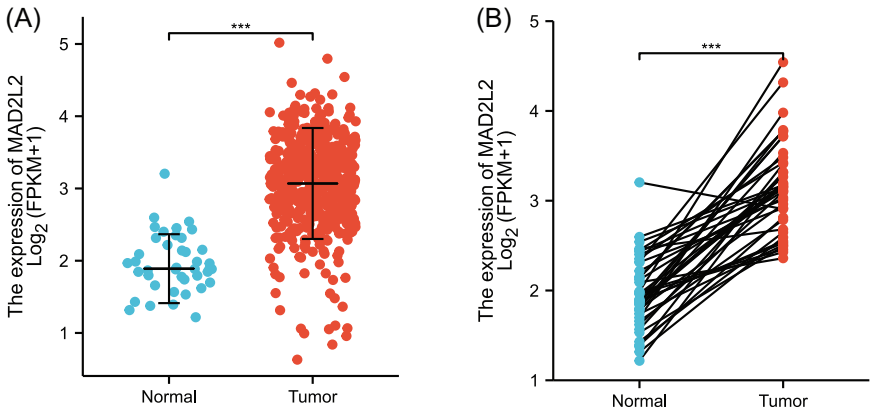

FIGURE 2. *MAD2L2* expression in adenocarcinoma of the colon tissues. (A) Expression of *MAD2L2* in normal and malignant tissues. (B) Expression of *MAD2L2* in matched tissues. ***, $p < 0.001$.

TABLE 2

Logistic analysis of the association between MAD2L2 expression and clinical characteristics

Characteristics	Total (N)	Odds ratio (OR)	*p* value
Gender (Male *vs.* Female)	478	1.309 (0.914–1.877)	0.143
Age (>65 *vs.* <=65)	478	1.275 (0.885–1.841)	0.193
T stage (T3&T4 *vs.* T1&T2)	477	0.813 (0.515–1.277)	0.370
N stage (N1&N2 *vs.* N0)	478	0.870 (0.604–1.254)	0.456
M stage (M1 *vs.* M0)	415	0.719 (0.421–1.219)	0.222
Pathologic stage (Stage III & Stage IV *vs.* Stage I & Stage II)	467	0.802 (0.555–1.158)	0.240
Primary therapy outcome (PR&CR *vs.* PD&SD)	250	1.841 (0.842–4.195)	0.133
Residual tumor (R1&R2 *vs.* R0)	374	0.912 (0.419–1.984)	0.814
CEA level (>5 *vs.* <=5)	303	1.040 (0.649–1.667)	0.871
Perineural invasion (YES *vs.* NO)	181	0.907 (0.443–1.809)	0.784
Lymphatic invasion (YES *vs.* NO)	434	1.263 (0.858–1.862)	0.237
History of colon polyps (YES *vs.* NO)	408	2.314 (1.531–3.525)	<0.001
Colon polyps present (YES *vs.* NO)	249	1.214 (0.706–2.079)	0.480

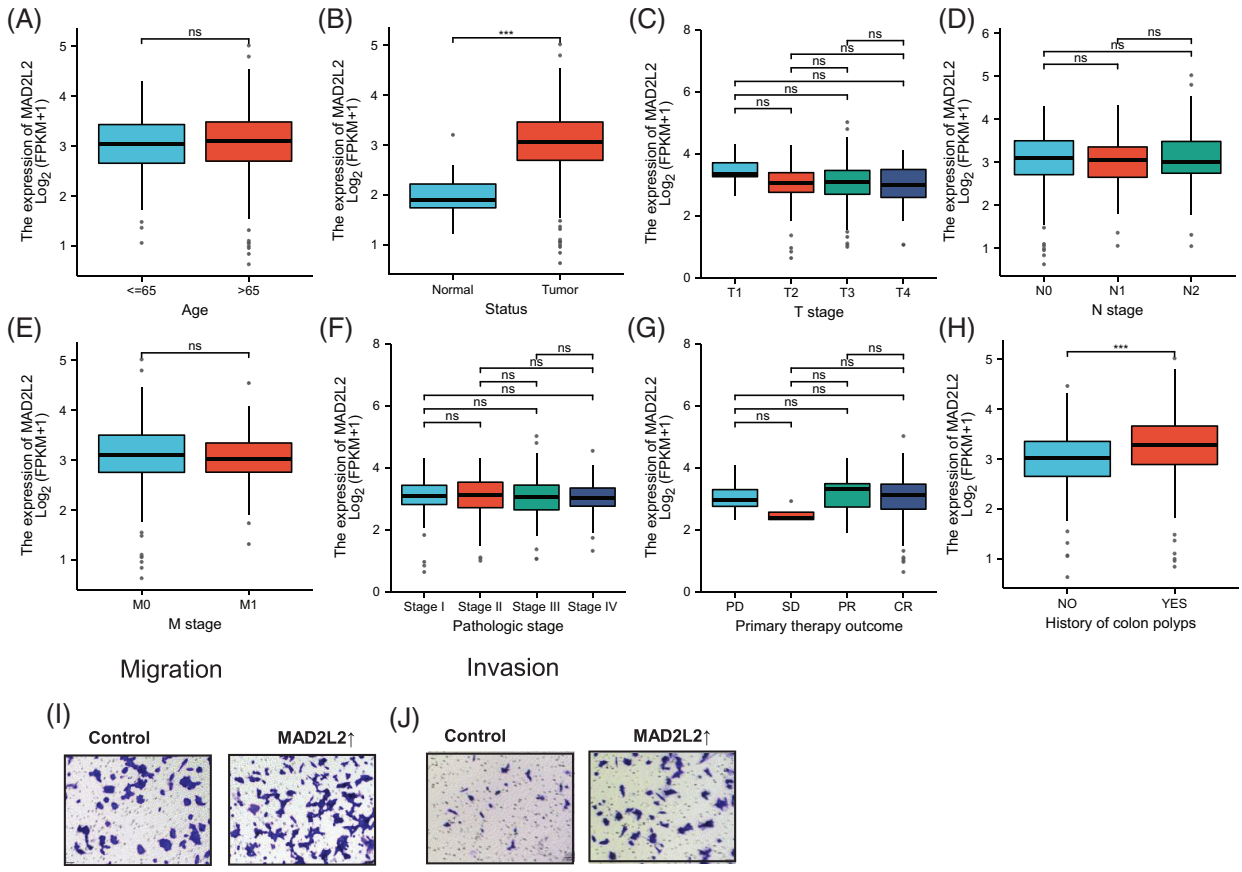

FIGURE 3. *MAD2L2* expression of patients with COAD according to different clinical characteristics. (A) Age; (B) Status; (C) T stage; (D) N stage; (E) M stage; (F) Pathologic stage; (G) Primary therapy outcome; (H) History of colon polyps; (I) Migration of HCT116 cells in control group and *MAD2L2* high expression group; (J) Invasion of HCT116 cells in control group and *MAD2L2* high expression group. ns, $p \geq 0.05$; ***, $p < 0.001$.

gene expression was diagnostic in different stages of COAD, with AUC values of 0.922 in paracancerous tissue *vs.* stage I, 0.744 in T1/T2, 0.699 in T1/T3, 0.744 in T1/T4, and 0.744 in paracancerous tissue *vs.* N0 stage, paracancerous tissue *vs.* M0 stage, and paracancerous tissue *vs.* M1 stage being the most significant (Figs. 4B–4H). The results showed that MAD2L2 has certain diagnostic value in different clinical stages. We developed a nomogram to predict the 1-, 3-, and 5-year survival probability of patients with COAD by integrating the MAD2L2 gene expression level with

TABLE 3

Univariate and multivariate Cox regression analyses of clinical characteristics associated with overall survival

Characteristics	Total (N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*p* value	Hazard ratio (95% CI)	*p* value
MAD2L2	477	1.105 (0.795–1.537)	0.553		
Gender	477				
Female	226	Reference			
Male	251	1.101 (0.746–1.625)	0.627		
Age	477				
<=65	194	Reference			
>65	283	1.610 (1.052–2.463)	**0.028**	0.001 (0.000-Inf)	1.000
T stage	476				
T1	11	Reference			
T2	83	0.417 (0.080–2.159)	0.297	574593520265823.750 (0.000-Inf)	1.000
T3	322	1.291 (0.316–5.268)	0.722	48719.583 (0.000-Inf)	1.000
T4	60	3.508 (0.818–15.037)	0.091	44.822 (0.000-Inf)	1.000
N stage	477				
N0	283	Reference			
N1	108	1.681 (1.019–2.771)	**0.042**	135118.696 (0.000-Inf)	1.000
N2	86	4.051 (2.593–6.329)	**<0.001**	108246937136642.031 (0.000-Inf)	1.000
M stage	414				
M0	348	Reference			
M1	66	4.193 (2.683–6.554)	**<0.001**	0.066 (0.000-Inf)	1.000
Pathologic stage	466				
Stage I	81	Reference			
Stage II	186	2.035 (0.785–5.273)	0.143	6961814585.362 (0.000-Inf)	1.000
Stage III	133	3.683 (1.436–9.448)	**0.007**	67.731 (0.000-Inf)	1.000
Stage IV	66	9.294 (3.608–23.936)	**<0.001**	1.000 (0.000-Inf)	1.000
CEA level	302				
<=5	195	Reference			
>5	107	3.128 (1.788–5.471)	**<0.001**	6871.753 (0.000-Inf)	1.000
History of colon polyps	407				
NO	262	Reference			
YES	145	0.741 (0.442–1.242)	0.255		

characteristics related with clinical prognosis and diagnosis (Fig. 5). The score could be used by doctors to forecast patients' 1-, 3-, and 5-year survival rates. These findings indicate that MAD2L2 could be utilized as a predictor in the diagnosis of COAD at various clinical stages.

MAD2L2 affects the expression of infiltrating immune cells in COAD

Tumor-infiltrating immune cells play a key role in predicting overall survival in patients with COAD. Therefore, using TIMER, we analyzed the correlation between MAD2L2 expression and immune infiltration levels in COAD. As shown in Fig. 6A, MAD2L2 expression was positively correlated with neutrophil levels ($p = 7.4 \times 10^{-4}$). The results suggest that MAD2L2 plays a role in the immune infiltration of COAD. Additionally, we aimed to evaluate whether the tumor immune microenvironment of patients with COAD who expressed high levels of MAD2L2 was distinct from that of patients with COAD who expressed low levels of MAD2L2. The 480 COAD samples were divided into two groups based on MAD2L2 expression, with 240 samples classified as high expression and 240 as low expression. Using the R package, we performed calculations on immune infiltration to determine levels of 24 immune cells. The immune infiltration algorithm applied to 24 immune cell subtypes helped to assess differences in immune cell expression levels between the high and low MAD2L2 expression groups (Fig. 6B). T cells, aDCs, cytotoxic cells, DCs, iDCs, neutrophils, NK CD56 bright cells, NK CD56dim cells, Tcm cells, Th1 cells, Th17 cells, and Tregs were significantly affected by MAD2L2 expression. Compared with the low expression group, T

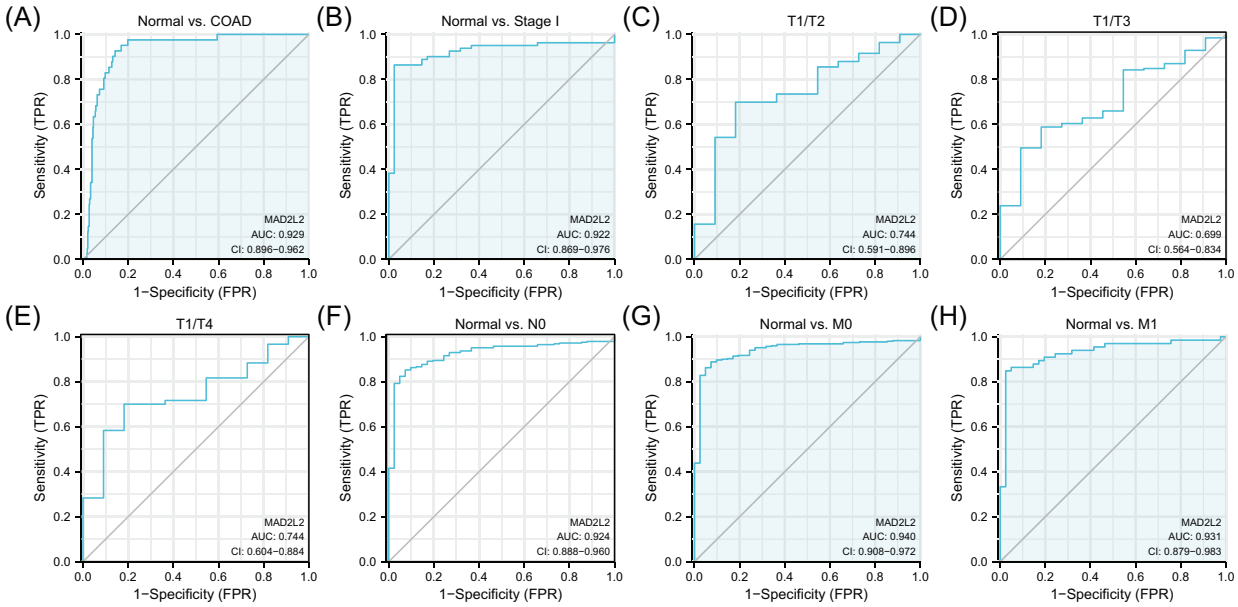

FIGURE 4. Diagnostic value of *MAD2L2* expression in colon cancer. (A) ROC curve for *MAD2L2* in normal colon tissue and COAD; (B–H) Subgroup analysis for Normal *vs.* stage I, T1/T2, T1/T3, T1/T4, Normal *vs.* N0, Normal *vs.* M0, Normal *vs.* M1.

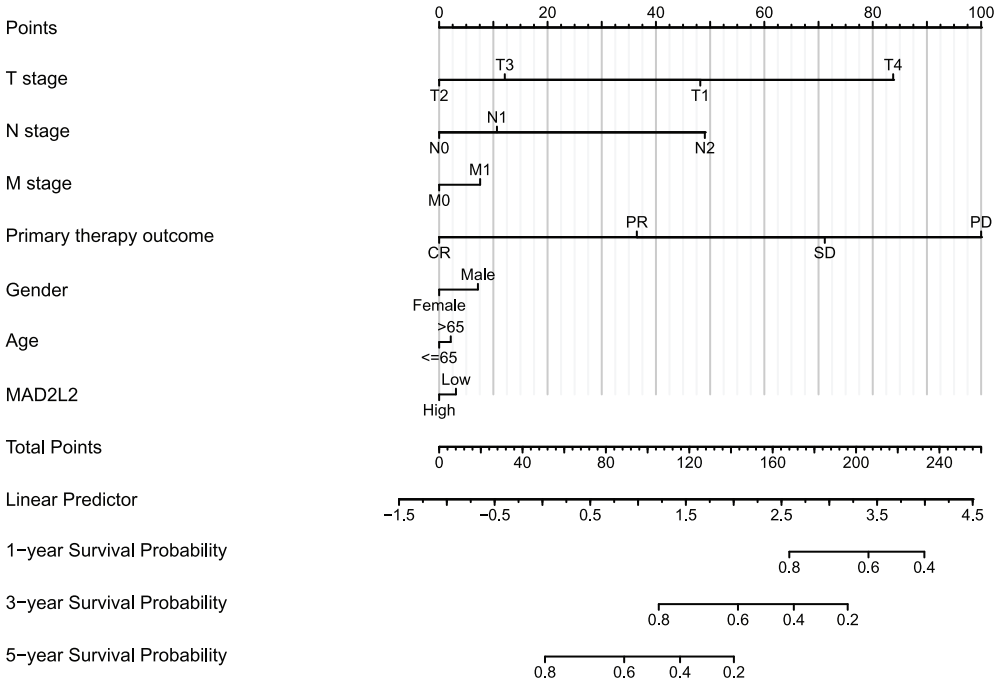

FIGURE 5. Nomogram used to predict the probability of patients with 1-, 3-, and 5-year overall survival. For risk estimation, determine the status of each clinical factor and the expression value of *MAD2L2*, and draw a straight line up to the point axis to view the points generated by a single factor. Repeat until the score of all factors is determined. Sum the points and find the summation point on the total points axis. The 1-, 3- and 5-year survival probabilities were then obtained by plotting straight down to the risk axis.

cells, aDCs, cytotoxic cells, DCs, iDCs, neutrophils, NK CD56 bright cells, NK CD56dim cells, Th1 cells, Th17 cells, and TReg increased in the high expression group ($p < 0.05$), while Tcm decreased ($p < 0.05$). We also assessed possible correlations between 24 immune cells (Fig. 6C). The generated heatmap revealed associations between the ratios of several tumor-infiltrating immune cell groups ranging from weak to robust. Also, we validated it at the protein level. Using different concentrations of TNF-α acting on colon cancer HCT116 cells, MAD2L2 showed a dose-dependent effect as the concentration of TNF-α increased (Figs. 7A and 7B). As a result, we hypothesize that MAD2L2 expression is associated with tumor immune infiltration throughout the development of COAD and, to a lesser degree, influences immune cell expression in the tumor immune microenvironment.

Biological function of MAD2L2 in COAD

To understand the biological significance of *MAD2L2* in COAD, we used the R package to analyze the co-expression network of *MAD2L2* in COAD data. Based on our findings, we screened and examined the influence of the expression of the top 50 genes positively related with *MAD2L2*, against the top 50 genes negatively associated with *MAD2L2* expression, on overall survival in colon cancer. The results are shown in the heat map in Figs. 8A and 8B. Genetic risk for COAD is predicted to be highest in the top 50 positively associated genes. The top 50 negatively related genes, on the

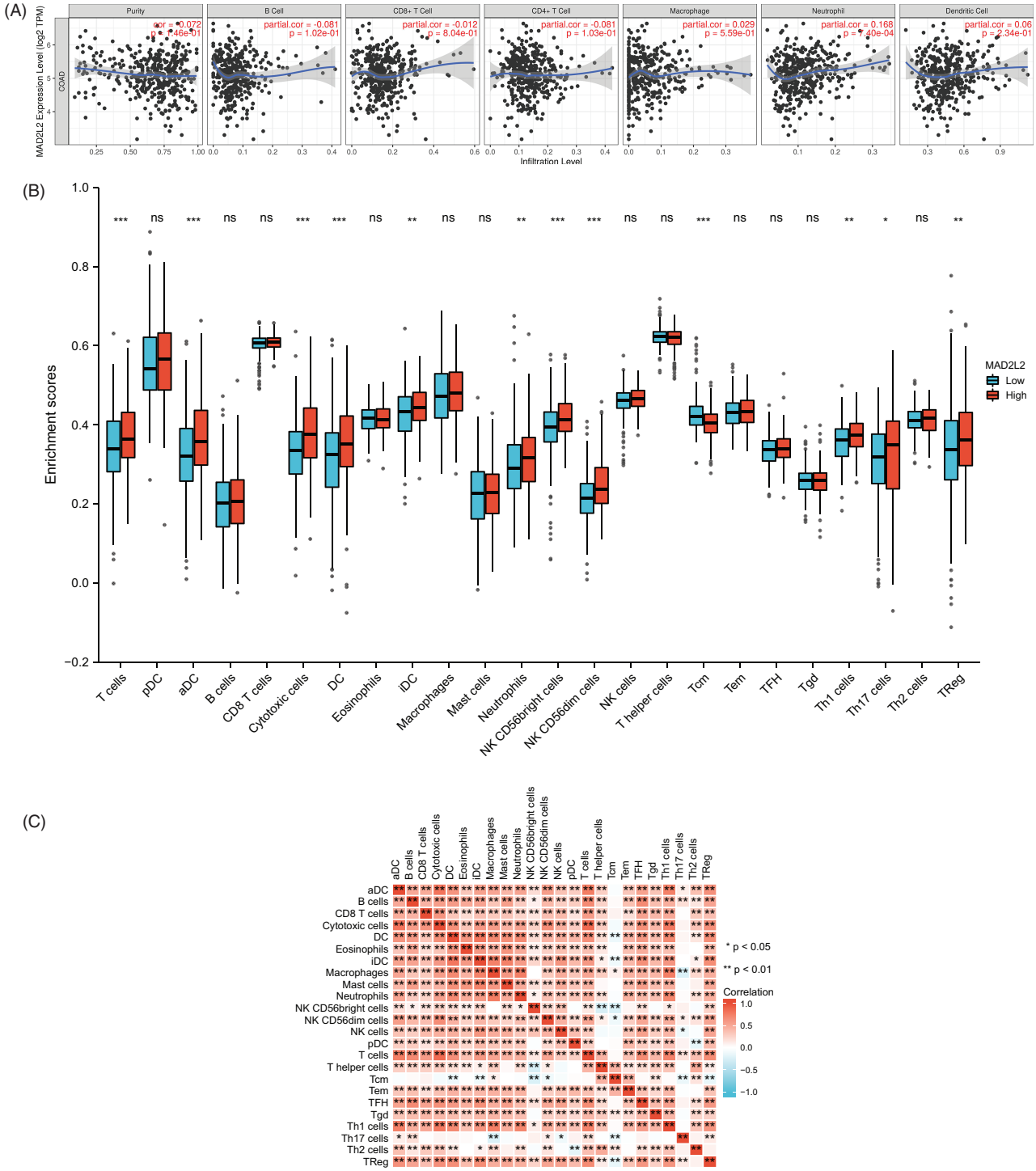

FIGURE 6. *MAD2L2* expression and immunological infiltration are correlated. (A) Correlations between *MAD2L2* expression and infiltration levels of the immune system. (B) The proportions of 24 immune cell subtypes in colon cancer samples with high and low *MAD2L2* expression. (C) Heatmap depicting the invasion of 24 immune cells into colon cancer samples. ns, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

other hand, were the ones that were most likely to be low-risk genes for COAD, according to the analysis. Additionally, this shows that *MAD2L2*, along with other genes, may be implicated in the overexpression of COAD risk factors and the downregulation of COAD preventative factors, as well as in the occurrence and development of COAD. The Kyoto Encyclopedia of Genes and Genomes (KEGG) Gene Ontology (GO) term annotations showed that MAD2L2 was mainly involved in biological processes when enriched with positively correlated genes, including response to hypoxia, response to decreased oxygen levels, anaphase-promoting complex-dependent catabolic process, response to oxygen levels, mitochondrial translational elongation, cellular components including vesicle coat, Fanconi anemia nuclear complex, ribosome, mitochondrial matrix, mitochondrial inner membrane, pathways including Huntington's disease and Parkinson's disease, ubiquitin mediated proteolysis, pathways of neurodegeneration-multiple diseases (Fig. 8C). Histone modification and covalent chromatin modification were inhibited, as were molecular functions such as histone

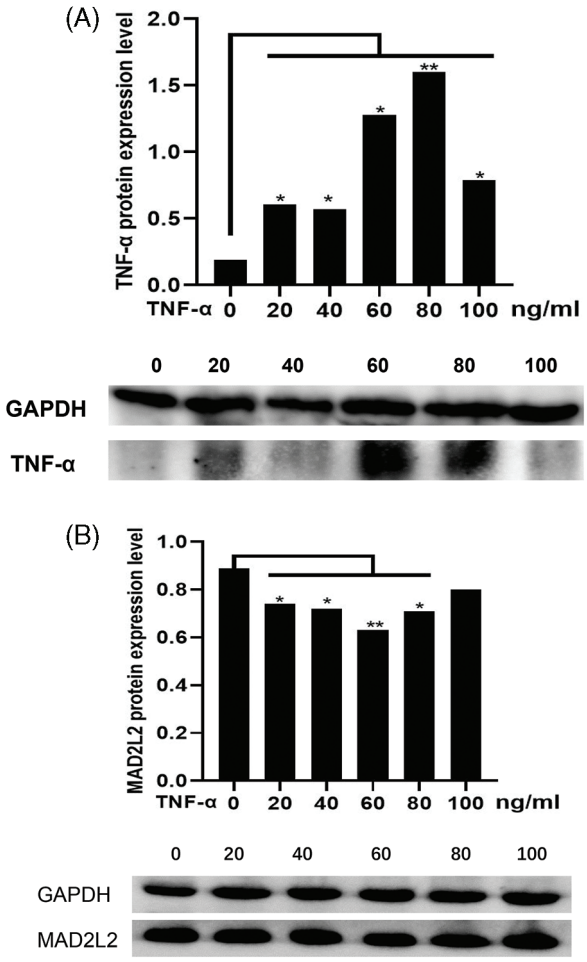

FIGURE 7. Effect of TNF-α stimulation on *MAD2L2* expression. (A) Effect of TNF-α protein expression in COAD under different concentrations of TNF-α. (B) Effect of *MAD2L2* protein expression in COAD under different concentrations of TNF-α. *, $p < 0.05$; **, $p < 0.01$.

binding, modification-dependent protein binding, histone demethylase activity (H3-K9 specific), methylation-dependent protein binding, and lysine degradation. Biological processes such as histone modification and covalent chromatin modification were also inhibited, as well as pathways such as lysine degradation, which were all inhibited as well (Fig. 8D). From these results, we infer that in COAD, processes such as the hypoxia response, response to reduced oxygen levels, and mitochondrial translation elongation are closely related to *MAD2L2* expression.

Discussion

Approximately 70%–80% of colorectal cancers are sporadic tumors (Fearon and Vogelstein, 1990), and their occurrence and development mostly follow the sequence of "adenoma-cancer". It generally takes 5 to 10 years for the progression from precancerous lesions to cancer, which is the early disease stage. Diagnosis and clinical intervention provide important time windows (Brody, 2015). According to research, colorectal cancer screening, early detection, and treatment may significantly decrease the mortality of individuals with colorectal cancer (Lin *et al.*, 2021). The current work established MAD2L2's diagnostic and prognostic relevance in COAD, the processes behind its development, and its relationship with immune infiltration and mutation accumulation in hypoxic conditions.

In recent years, many genes important for colorectal cancer progression have been identified (Mo *et al.*, 2015; Kundu *et al.*, 2021; Smeby *et al.*, 2020; Golla *et al.*, 2020; Voutsadakis, 2021; Liu *et al.*, 2019a, 2019b), including KRAS, TP53, APC, PIK3CA, PPARD, and MORC2. With the continuous development of high-throughput technology, microarray analysis has been used to identify unknown colorectal cancer-related oncogenes and biological network analysis (Bogaert and Prenen, 2014). An article published in 2007 indicated that increased MAD2L2 expression was related with a worse outcome in colorectal cancer (Rimkus *et al.*, 2007). Tumors with high expression of MAD2L2 show a marked increase in abnormal mitosis, resulting in chromosomal instability (Varadi *et al.*, 2011). Aberrant expression of MAD2L2 disrupts genome integrity and leads to cancer (Dev *et al.*, 2018). MAD2L2 overexpression promotes the proliferation of human breast cancer cells by promoting abnormal mitosis (Yuan *et al.*, 2006). High MAD2L2 expression is associated with significantly shorter overall survival and progression-free survival in patients with diffuse large B-cell lymphoma (Okina *et al.*, 2015). Consistent with these studies, the results of our RNA-seq data analysis confirmed that expression of MAD2L2 was higher in COAD cancer tissues than in paracancerous tissue in unpaired samples and paired samples, and the mRNA expression of MAD2L2 was significantly upregulated, suggesting that MAD2L2 may be involved in COAD and play an important role in its occurrence and development. In addition, the ROC curve results indicated that MAD2L2 has a high diagnostic value in COAD prognosis.

A study of human colorectal cancer transcripts found that MAD2L2 is not commonly upregulated in colorectal benign adenomas, and its upregulation may represent a relatively late event in the carcinogenesis process (Rimkus *et al.*, 2007). Consistent with this, our investigation discovered a substantial association between MAD2L2 expression and a history of colonic polyps. Meanwhile, in the results of our univariate association analysis, we showed that high MAD2L2 expression was significantly and negatively associated with NCOA3. NCOA3 is a transcriptional co-activator with elevated expression in several tumor types including CRC, which was shown to promote CRC progression by enhancing the Notch signaling pathway (Mo *et al.*, 2015). According to research conducted by Li *et al.* (2018) overexpression of MAD2L2 may reduce the proliferation, migration, and clonogenicity of CRC cells by triggering the degradation of NCOA3 in the cells. It is suggested that the upregulation of MAD2L2 may not be the cause but a passive consequence of the unstable cancer cell genome adjusting to maintain stability. Cancer-promoting factors such as CHD9, UBN2, and CPEB2, which also support this view, were significantly negatively correlated with the high expression of MAD2L2. When DNA damage occurs, POL3 is normally required in normal cells to complete repair. In contrast to normal cells, cancer cells

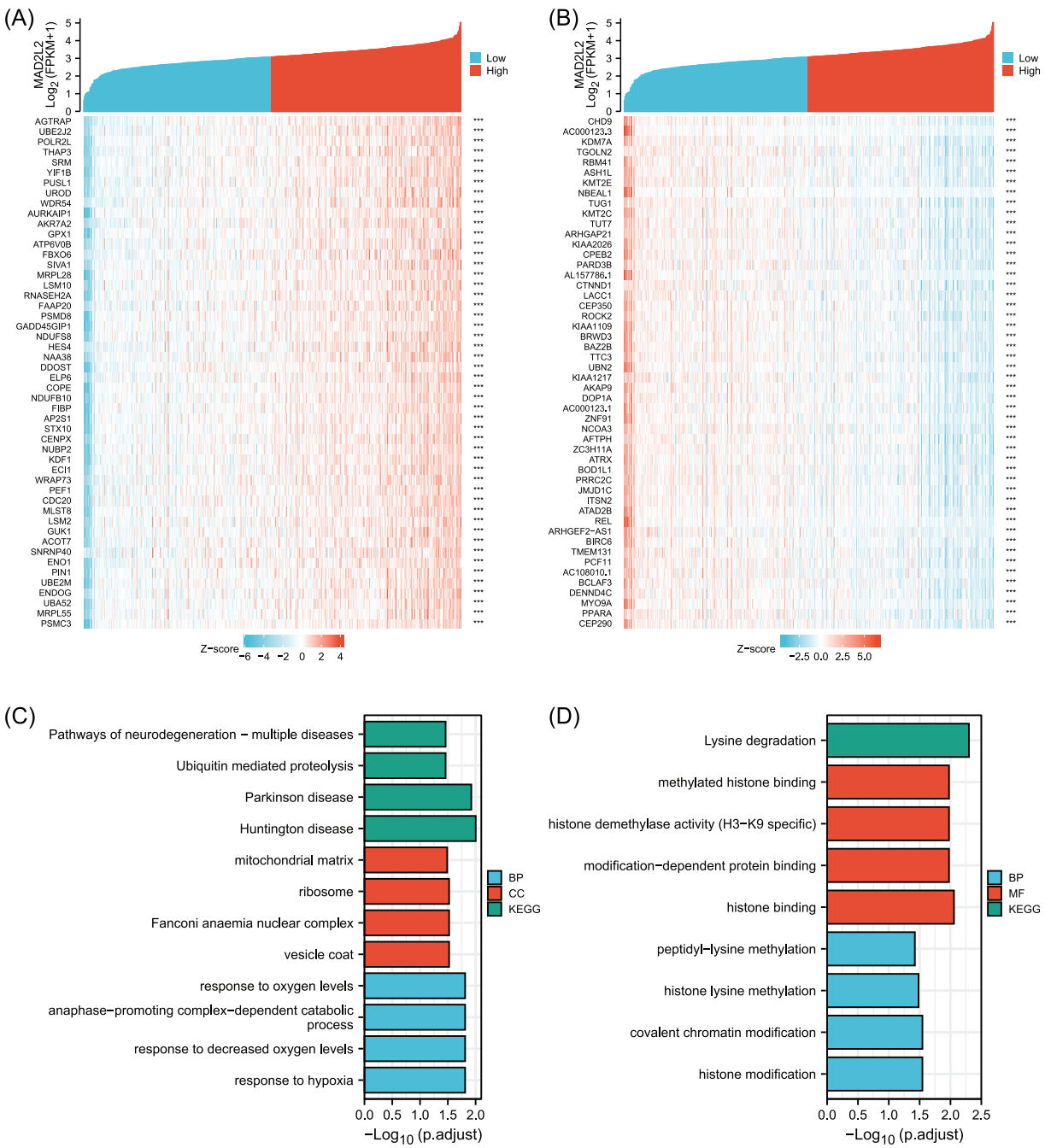

FIGURE 8. Genes coexpressed with *MAD2L2* in COAD. (A) The top 50 positively correlated genes cotranscript with *MAD2L2* in COAD. (B) The top 50 negatively correlated genes cotranscript with *MAD2L2* in COAD. (C) BP, CC and KEGG pathway analysis of *MAD2L2* positively related genes in COAD. (D) BP, MF and KEGG pathway analysis of *MAD2L2* negatively correlated genes in COAD. ***, $p < 0.001$.

generally have a penchant for relying on error-repair bypasses, especially the TLS repair bypass, and this pathway is commonly performed by the error-prone and more efficient POLζ, of which MAD2L2 is a subunit (Stodola *et al.*, 2016). We hypothesize that the passive high expression of MAD2L2 in COAD contributes to genomic instability and mutational load tolerance in cancer cells.

MAD2L2 is primarily engaged in biological activities when overexpressed, including the hypoxia response, reaction to decreased oxygen levels, mitochondrial translation elongation, and other processes, as determined by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) investigations. PRDX2, a typical 2-Cys antioxidant enzyme, plays an important role in scavenging hydrogen peroxide and reactive oxygen species (ROS) levels, thereby protecting cells from oxidative stress (de Franceschi *et al.*, 2011). MAD2L2 can respond to changes in radiation-generated ROS levels by recruiting PRDX2 (Gu *et al.*, 2019). Our findings indicate that MAD2L2 expression is positively linked with PRDX2 expression, and that the glycolytic pathway works as an energy source in cancer cells, resulting in compromised mitochondrial activity and long-term cellular exposure to the potential hazard of ROS. We speculate that MAD2L2 may cooperate with PRDX2 to scavenge overloaded hydrogen peroxide and ROS levels to maintain cancer cell homeostasis. This is more proof supporting MAD2L2's passive impact.

Equally important, we used the TIMER database to reveal the correlation between MAD2L2 expression and immune infiltration in COAD. We found that MAD2L2 expression was positively correlated with neutrophil levels. When MAD2L2 is highly expressed, T cells, aDCs, cytotoxic cells, DCs, iDCs, neutrophils, NK CD56 bright cells, NK CD56 dim cells, Th1 cells, Th17 cells, and TReg increase. Studies have found that neutrophils are early triggers of various injuries, and their infiltration in intestinal lesions is a marker of the acute phase of ulcerative colitis (Dinallo *et al.*, 2019). Neutrophil elastase, released by neutrophils, is involved in the body's immune defense and inflammatory response. Elevated levels of neutrophil elastase are highly correlated with COAD invasion and metastasis (Huang *et al.*, 2020). We reasoned that MAD2L2 overexpression might promote neutrophil immune responses and infiltration, thereby triggering tumor immune responses. These results indicate that MAD2L2 expression is required for the control and recruitment of invading immune cells throughout the formation and progression of COAD. However, more cell experiments and the accumulation of clinical data are needed to more accurately understand the interaction between MAD2L2 and neutrophils.

Given that MAD2L2 is significantly expressed in COAD, its increased expression is strongly associated with a poor clinical prognosis and has a high diagnostic value in COAD, suggesting that it may be employed as an independent prognostic factor for COAD patient survival. Additionally, we discovered a strong correlation between MAD2L2 expression and tumor-infiltrating immunity. A further discovery was made using gene set enrichment analysis, which revealed that MAD2L2 has a broad range of potential applications in the occurrence and development of COAD. This is the first study to assess the prognosis of patients with colon cancer from all directions when MAD2L2 expression is abnormal. With a greater knowledge of MAD2L2's functional range, it may serve as a useful target factor for the clinical diagnosis and prognosis of COAD, perhaps paving the way for biomarker therapy to become the treatment of choice for COAD in the future. Furthermore, even if MAD2L2 can be applied to guide clinical practice in the future, other molecules that interact with MAD2L2 remain to be discovered because it is not a single gene or marker but a set of comprehensive molecular marker signatures.

Availability of Data and Materials: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contribution: HTS and FX designed the study. HYW performed table production. XL contributed to image drawing. YJH and JL performed download data. FMW and HTS acquired and interpretated the data. HW and HTS assisted with data analysis. All authors read and approved the final manuscript.

Ethics Approval: Not applicable.

Funding Statement: The present study was supported by the Ningxia Hui Autonomous Region Key Research and Development Program (Grant No. 2021BEG03084).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

References

Ben S, Zhu Q, Chen S, Li S, Du M, Xin J, Chu H, Zhang Z, Wang M (2021). Genetic variations in the CTLA-4 immune checkpoint pathway are associated with colon cancer risk, prognosis, and immune infiltration via regulation of IQCB1 expression. *Archives of Toxicology* **95**: 2053–2063. DOI 10.1007/s00204-021-03040-0.

Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M et al. (2013). Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* **39**: 782–795. DOI 10.1016/j.immuni.2013.10.003.

Bogaert J, Prenen H (2014). Molecular genetics of colorectal cancer. *Annals of Gastroenterology* **27**: 9–14.

Brody H (2015). Colorectal cancer. *Nature* **521**: S1. DOI 10.1038/521S1a.

de Franceschi L, Bertoldi M, de Falco L, Santos Franco S, Ronzoni L, Turrini F, Colancecco A, Camaschella C, Cappellini MD, Iolascon A (2011). Oxidative stress modulates heme synthesis and induces peroxiredoxin-2 as a novel cytoprotective response in β-thalassemic erythropoiesis. *Haematologica* **96**: 1595–1604. DOI 10.3324/haematol.2011.043612.

de Krijger I, Boersma V, Jacobs JJL (2021). REV7: Jack of many trades. *Trends in Cell Biology* **31**: 686–701. DOI 10.1016/j.tcb.2021.04.002.

Dev H, Chiang TW, Lescale C, de Krijger I, Martin AG et al. (2018). Shieldin complex promotes DNA end-joining and counters homologous recombination in BRCA1-null cells. *Nature Cell Biology* **20**: 954–965. DOI 10.1038/s41556-018-0140-1.

Dinallo V, Marafini I, di Fusco D, Laudisi F, Franzè E et al. (2019). Neutrophil extracellular traps sustain inflammatory signals in ulcerative colitis. *Journal of Crohn's and Colitis* **13**: 772–784. DOI 10.1093/ecco-jcc/jjy215.

Dulskas A, Gaizauskas V, Kildusiene I, Samalavicius NE, Smailyte G (2020). Improvement of survival over time for colorectal cancer patients: A population-based study. *Journal of Clinical Medicine* **9**: 4038. DOI 10.3390/jcm9124038.

Fang X, Hong Y, Dai L, Qian Y, Zhu C, Wu B, Li S (2017). CRH promotes human colon cancer cell proliferation via IL-6/JAK2/STAT3 signaling pathway and VEGF-induced tumor angiogenesis. *Molecular Carcinogenesis* **56**: 2434–2445. DOI 10.1002/mc.22691.

Fearon ER, Vogelstein B (1990). A genetic model for colorectal tumorigenesis. *Cell* **61**: 759–767. DOI 10.1016/0092-8674(90)90186-I.

Feng L, Wei W, Heng Z, Yantao H, Chunbo W (2016). Knockdown of REV7 inhibits breast cancer cell migration and invasion. *Oncology Research* **24**: 315–325. DOI 10.3727/096504016X14666990347590.

Golla JP, Kandyliari A, Tan WY, Chen Y, Orlicky DJ, Thompson DC, Shah YM, Vasiliou V (2020). Interplay between APC and ALDH1B1 in a newly developed mouse model of colorectal cancer. *Chemico-Biological Interactions* **331**: 109274. DOI 10.1016/j.cbi.2020.109274.

Gu C, Luo J, Lu X, Tang Y, Ma Y et al. (2019). REV7 confers radioresistance of esophagus squamous cell carcinoma by recruiting PRDX2. *Cancer Science* **110**: 962–972. DOI 10.1111/cas.13946.

Hong CS, Sun EG, Choi JN, Kim DH, Kim JH et al. (2020). Fibroblast growth factor receptor 4 increases epidermal growth factor

receptor (EGFR) signaling by inducing amphiregulin expression and attenuates response to EGFR inhibitors in colon cancer. *Cancer Science* **111**: 3268–3278. DOI 10.1111/cas.14526.

Huang H, Zhang H, Onuma AE, Tsung A (2020). Neutrophil elastase and neutrophil extracellular traps in the tumor microenvironment. *Advances in Experimental Medicine and Biology* **1263**: 13–23. DOI 10.1007/978-3-030-44518-8.

Karakashev S, Fukumoto T, Zhao B, Lin J, Wu S et al. (2020). EZH2 inhibition sensitizes CARM1-high, homologous recombination proficient ovarian cancers to PARP inhibition. *Cancer Cell* **37**: 157–167.e6. DOI 10.1016/j.ccell.2019.12.015.

Kundu S, Ali MA, Handin N, Conway LP, Rendo V, Artursson P, He L, Globisch D, Sjöblom T (2021). Common and mutation specific phenotypes of KRAS and BRAF mutations in colorectal cancer cells revealed by integrative-omics analysis. *Journal of Experimental & Clinical Cancer Research* **40**: 225. DOI 10.1186/s13046-021-02025-2.

Li Y, Li L, Chen M, Yu X, Gu Z et al. (2018). MAD2L2 inhibits colorectal cancer growth by promoting NCOA3 ubiquitination and degradation. *Molecular Oncology* **12**: 391–405. DOI 10.1002/1878-0261.12173.

Lin JS, Perdue LA, Henrikson NB, Bean SI, Blasi PR (2021). Screening for colorectal cancer: Updated evidence report and systematic review for the US preventive services task force. *Journal of the American Medical Association* **325**: 1978–1998. DOI 10.1001/jama.2021.4417.

Liu Y, Deguchi Y, Tian R, Wei D, Wu L et al. (2019a). Pleiotropic effects of PPARD accelerate colorectal tumorigenesis, progression, and invasion. *Cancer Research* **79**: 954–969. DOI 10.1158/0008-5472.CAN-18-1790.

Liu J, Shao Y, He Y, Ning K, Cui X, Liu F, Wang Z, Li F (2019b). MORC2 promotes development of an aggressive colorectal cancer phenotype through inhibition of NDRG1. *Cancer Science* **110**: 135–146. DOI 10.1111/cas.13863.

Malayaperumal S, Sriramulu S, Banerjee A, Makalakshmi MK, Pathak S (2021). Is biotechnological next-generation therapeutics promising enough in clinical development to treat advanced colon cancer? *Current Pharmaceutical Biotechnology* **22**: 1287–1301. DOI 10.2174/1389201021666201126142716.

Mo P, Zhou Q, Guan L, Wang Y, Wang W et al. (2015). Amplified in breast cancer 1 promotes colorectal cancer progression through enhancing notch signaling. *Oncogene* **34**: 3935–3945. DOI 10.1038/onc.2014.324.

Okina S, Yanagisawa N, Yokoyama M, Sakurai Y, Numata Y, Umezawa A, Higashihara M, Murakumo Y (2015). High expression of REV7 is an independent prognostic indicator in patients with diffuse large B-cell lymphoma treated with rituximab. *International Journal of Hematology* **102**: 662–669. DOI 10.1007/s12185-015-1880-3.

Rimkus C, Friederichs J, Rosenberg R, Holzmann B, Siewert JR, Janssen KP (2007). Expression of the mitotic checkpoint gene MAD2L2 has prognostic significance in colon cancer. *International Journal of Cancer* **120**: 207–211. DOI 10.1002/(ISSN)1097-0215.

Saito Y, Koya J, Kataoka K (2021). Multiple mutations within individual oncogenes. *Cancer Science* **112**: 483–489. DOI 10.1111/cas.14699.

Smeby J, Kryeziu K, Berg KCG, Eilertsen IA, Eide PW et al. (2020). Molecular correlates of sensitivity to PARP inhibition beyond homologous recombination deficiency in pre-clinical models of colorectal cancer point to wild-type TP53 activity. *eBioMedicine* **59**: 102923. DOI 10.1016/j.ebiom.2020.102923.

Stodola JL, Stith CM, Burgers PM (2016). Proficient replication of the yeast genome by a viral DNA polymerase. *MOJ Bioorganic & Organic Chemistry* **291**: 11698–11705. DOI 10.1074/jbc.M116.728741.

Varadi V, Bevier M, Grzybowska E, Johansson R, Enquist K et al. (2011). Genetic variation in genes encoding for polymerase ζ subunits associates with breast cancer risk, tumour characteristics and survival. *Breast Cancer Research and Treatment* **129**: 235–245. DOI 10.1007/s10549-011-1460-z.

Voutsadakis IA (2021). The landscape of PIK3CA mutations in colorectal cancer. *Clinical Colorectal Cancer* **20**: 201–215. DOI 10.1016/j.clcc.2021.02.003.

Weiser MR (2018). AJCC 8th edition: Colorectal cancer. *Annals of Surgical Oncology* **25**: 1454–1455. DOI 10.1245/s10434-018-6462-1.

Yu Z, Li X, Duan J, Yang XD (2020). Targeted treatment of colon cancer with aptamer-guided albumin nanoparticles loaded with docetaxel. *International Journal of Nanomedicine* **15**: 6737–6748. DOI 10.2147/ijn.S267177.

Yuan B, Xu Y, Woo JH, Wang Y, Bae YK, Yoon DS, Wersto RP, Tully E, Wilsbach K, Gabrielson E (2006). Increased expression of mitotic checkpoint genes in breast cancer cells with chromosomal instability. *Clinical Cancer Research* **12**: 405–410. DOI 10.1158/1078-0432.CCR-05-0903.

Zhang Y, Li X, Zhou D, Zhi H, Wang P et al. (2018). Inferences of individual drug responses across diverse cancer types using a novel competing endogenous RNA network. *Molecular Oncology* **12**: 1429–1446. DOI 10.1002/1878-0261.12181.

Zhao T, Feng Y, Guo M, Zhang C, Wu Q et al. (2020). Combination of attenuated Salmonella carrying PD-1 siRNA with nifuroxazide for colon cancer therapy. *Journal of Cellular Biochemistry* **121**: 1973–1985. DOI 10.1002/jcb.29432.