DEFB4A is a potential prognostic biomarker for colorectal cancer

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Abstract. Colorectal cancer (CRC) is the third leading cause of cancer-associated mortality. The present study aimed to investigate novel biomarkers to predict prognosis and provide a theoretical basis for studies of the pathogenesis and the development of therapies for CRC. The present study compared mRNA expression levels of patients with CRC with short- and long-term prognosis and of individuals with and without tumors in The Cancer Genome Atlas (TCGA) database. Differentially expressed genes (DEGs) were identified via volcano plot and Venn diagram analysis. Gene Ontology (GO) analysis and gene set enrichment analysis (GSEA) were performed to identify the functions of the DEGs, and the DEGs were further verified using clinical CRC samples. A total of 10 DEGs were identified as candidate genes using the TCGA database, and four DEGs [defensin β 4A (*DEFB4A*), hyaluronan binding protein 2 (*HABP2*), oleoyl-ACP hydrolase and TBC1 domain family member 3G] were associated with poor prognosis of patients with CRC. Two DEGs (*DEFB4A* and *HABP2*) were upregulated in tumor tissues of patients with CRC in the TCGA database. GO and GSEA analyses revealed that *DEFB4A* was highly associated with immunosuppression, participates in 'myeloid leukocyte differentiation', 'leukocyte proliferation' and 'positive regulation of leukocyte-mediated immunity', and was positively correlated with CD11b, CD14, CD45, CD163 and IL17A. Furthermore, *DEFB4A* expression was significantly upregulated in patients with large tumors, advanced cancer stage, lymph node metastasis and liver metastasis. Survival analysis revealed that *DEFB4A* upregulation was associated with poor prognosis. DEFB4A gene knockdown experiments demonstrated that DEF4BA promotes cell migration. These results indicated that *DEFB4A* potentially promotes tumor growth by regulating immunosuppressive activity and provided novel insights into the diagnosis and treatment of CRC.

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Introduction

According to the Global Cancer Statistic of 2020, colorectal cancer (CRC) is the third most common (9%) cancer and the second leading cause of cancer-associated mortality (9%) worldwide (1). CRC was reported as the fourth most common and fatal cancer in China, in 2008 (2). Patients with CRC usually have a low survival rate and poor therapeutic responses, and are susceptible to progression and recurrence (3). Early diagnosis and effective treatment are critical to improve the survival of patients with CRC (4). CRC studies have focused on innovative ideas to identify molecular markers used to develop high-precision, non-invasive screening tests for CRC to increase population compliance and reduce the potentially harmful side effects associated with more invasive techniques (5). Diagnostic markers will give an indication of the likely progression of the disease (6). Targeting specific molecules in certain patients has facilitated more personalized treatments that help prevent or decelerate cancer progression. The present study aimed to determine prognostic factors and novel therapeutic targets to improve the survival of patients with CRC.

Previous studies have focused on the identification of molecules associated with tumor progression through genetic or mRNA profiling and screening of patients with colon cancer (7-12). For example, the expression profiles of long non-coding RNAs (lncRNAs) were compared at specific tumor stages (T0, T1, T2 and T3) in an azoxymethane/dextran sodium sulfate-induced primary colon cancer model and upregulation of the lncRNA H19 predicted a poor prognosis (7). Other studies analyzed microRNA (miR) expression profiles between tumor tissues and matched non-tumor tissues obtained from patients with CRC. For example, miR-124 is significantly downregulated in tumor tissues and associated with poor survival of patients with CRC, and may thus be considered to be a poor prognostic marker of CRC (8,9). Furthermore, high expression levels of miR-203

and miR-21 in serum are associated with poor survival of patients with CRC (10,11). Analysis of The Cancer Genome Atlas (TCGA) database revealed that *MMP19* is upregulated in patients with CRC and is associated with tumor progression (12). However, to the best of our knowledge, no study has directly screened mRNA profiles based on prognosis. The present study divided patients with CRC into different groups based on prognosis and screened the mRNA profiles of the respective groups.

Defensin β 4A (*DEFB4A*), also known as *BD-2, SAP1, DEFB2, DEFB4, HBD-2, DEFB-2* and *DEFB102*, belongs to the defensin family comprising cytotoxic peptides secreted by neutrophils, which serve important roles in innate immune defense against microbial infections (13-15). *DEFB4A* is upregulated in cutaneous squamous cell carcinoma and basal cell carcinoma (16,17). It serves an important role in esophageal carcinogenesis both *in vivo* and *in vitro* (18). The genomic copy number of *DEFB4A* has been analyzed in 466 patients with Crohn's disease and 329 controls, and an elevated *DEFB4A* copy number has been identified as a risk factor for Crohn's disease regardless of disease origin (19). However, it remains unclear whether *DEFB4A* expression is associated with the prognosis of CRC. Furthermore, the role of *DEFB4A* in the immune system remains unclear.

The tumor microenvironment serves a significant role in tumor progression. Various immune elements comprise the tumor microenvironment, including bone marrow-derived cells, such as macrophages, $CD4^+$ T cells, $CD8^+$ T cells, B cells, natural killer cells and dendritic cells (20). Myeloid cells can differentiate into macrophages or myeloid-derived suppressor cells (MDSCs), which serve a tumorigenic role in the tumor microenvironment (21). MDSCs contribute to tumor vascular development by promoting angiogenesis and tumor growth (22). Tumor-associated macrophages (TAMs) are important regulators of tumorigenesis by inhibiting the anti-tumor effects of other cells, thus promoting tumor growth (23). However, it remains unclear whether *DEFB4A* has a regulatory effect on the tumor microenvironment or whether it promotes CRC progression.

To identify candidate target genes that potentially prolong patient survival, mRNA expression profiles of tissues from CRC samples were compared in the TCGA database. Venn analysis was performed to determine candidate genes upregulated in tumor tissues among patients with poor prognosis. Subsequently, immune-associated pathway enrichment was analyzed using Gene Ontology (GO) and gene set enrichment analysis (GSEA), and the correlations between candidate target genes and certain immune cells were determined. Finally, clinical samples and CRC cell lines were obtained to verify the clinical significance of the identified genes. The present results may provide insights into targeted therapy for CRC.

Materials and methods

Acquisition of microarray data. Microarray data were obtained from TCGA (http://cancergenome.nih.gov/) (24). RNA-seq data for 784 samples were included in the dataset (Project ID: TCGA-COADREAD), including 689 tumor samples from patients with CRC and 95 normal tissues from healthy donors.

Identification of differentially expressed genes (DEGs). TCGA data were divided into two groups based on different categories: Patient prognosis and gene expression in tumor and normal tissues. Venn analysis of the two groups was performed, and 10 genes associated with CRC prognosis were identified.

Venn analysis. To identify candidate genes associated with patient survival, the gene expression profiles in the two groups were analyzed using the Venn Diagram web tool (http://bioinformatics.psb.ugent.be/webtools/Venn).

GO analysis. Functional analysis of the DEGs was performed using GO (http://www.geneontology.org) based on biological processes (25).

GSEA. GSEA was conducted using GSEA v4.0.3 software (https://www.gsea-msigdb.org/gsea/index.jsp) and the gene used in the present study was downloaded from the Molecular Signatures Database (MSigDB, http://software. broadinstitute. org/gsea/msigdb/index.jsp, v4.0). MSigDB curates various gene sets, including 1,320 canonical signaling pathways from BioCarta (https://cgap.nci.nih.gov/cgap_mitelman_retire_notice.html), Kyoto Encyclopedia of Genes and Genomes (https://www.kegg.jp), PID (http://pid.nci.nih.gov), Reactome (https://reactome.org) and other pathway databases. TCGA data were analyzed via GSEA, and pathways with a false discovery rate (FDR) <0.05 were considered significant.

Patient characteristics. Tissue samples were obtained from 52 patients with CRC at The First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) between April 2013 and April 2014. Patients underwent surgical resection or colonoscopy and the samples were verified via pathological analysis. The clinical characteristics of the patients are shown in Table I. A total of 34 men and 18 women were included in the present study. The median age was 60 years (age range, 26-91 years). CRC was diagnosed by two pathologists on the basis of pathological assessment. The collection of specimens was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China; approval no. Science-2010-LW-1213), and informed consent was obtained from each patient with available follow-up information.

Reverse transcription-quantitative PCR. Total RNA was extracted from 52 pairs of tumor and normal tissue samples from patients with CRC using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA samples (1 μg) were incubated at 42˚C for 2 min, followed by incubation at 37˚C for 15 min and 85˚C for 5 sec, according to the reverse transcription reaction protocol (Takara Biotechnology Co., Ltd.). The conditions of PCR were as follows: 95˚C/10 min; 95˚C/10 sec, 60˚C/10 sec, 72˚C/10 sec, 40 cycles (Premix Ex Taq II, Roche). Target gene expression was simultaneously assessed relative to that of *GAPDH* (a housekeeping gene and internal control). The following primers were used: *DEFB4A* forward, 5'-CTC CTCTTCTCGTTCCTCTTCA-3' and reverse, 5'-GCAGGT AACAGGATCGCCTAT-3'; and *GAPDH* forward, 5'-GGA GCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTT

Table I. Characteristics of patients with colorectal carcinoma.

Characteristic	No. of cases	Percentage
Sex		
Male	34	65.4
Female	18	34.6
Age, years		
<60	26	50.0
≥60	26	50.0
Treatment		
Surgery	42	80.7
Others	10	19.3
Tumor size, mm		
<50	28	53.8
≥50	24	46.2
Pathological type		
Adenocarcinoma	47	90.4
Others	5	9.6
Lymph node metastasis		
Yes	19	36.5
No	33	63.5
TNM stage		
I	12	23.1
II	16	30.8
III	16	30.8
IV	8	15.3
Liver metastasis		
Negative	44	84.6
Positive	8	15.4
Differentiation		
Poor	12	23.1
Medium-well	40	76.9

'Others' include chemotherapy and radiotherapy.

GTCATACTTCTCATGG-3'. The present study compared the expression levels of the target genes in clinical samples using the $2^{-\Delta\Delta Cq}$ method (26). Expression levels of *DEFB4A* and *GAPDH* were examined for each sample and the relative expression levels of *DEFB4A* were determined using the $2^{-\Delta Cq}$ value of *DEFB4A* divided by that of *GAPDH* (26).

Cell transfection. SW480 and HCT116 cells were seeded into a 24-well plate. SW480 and HCT116 cells were purchased from Chinese Academy of Sciences Cell Bank and cultivated with DMEM-high glucose containing 10% FBS (Hyclone; GE Healthcare Life Sciences) and 1% penicillin-streptomycin, at 37˚C in 5% CO_2. The growth status of the cells was closely observed until they reached a fusion rate of 80%, and then cells were transfected with NC-small interfering RNA (negative control) (si-NC, sense: 5'-UUCUCCGAACGUGUC ACGUTT-3' and antisense: 5'-ACGUGACACGUUCGG AGAATT-3') and small interfering RNA targeting DEFB4A (si-DEFB4A) (si-DEFB4A, sense: 5'-UCCUCUUCAUAUUCC UGAUTT-3' and antisense: 5'-AUCAGGAAUAUGAAGAGG ATT-3') purchased from Shanghai GenePharma Co., Ltd. with jetPRIME Polyplus transfection reagent (Polyplus-transfection SA). After 24 h, the medium was changed to fresh medium, and cells were further incubated in 5% CO_2 for 48 h. Subsequently, cells were collected for the subsequent experiments.

Wound healing assay. For the wound healing assay, SW480 and HCT116 cells were cultured in 500 μl medium with 10% FBS (Hyclone) and the percentage of serum was in line with previous papers (27,28). Sub-confluent tumor cells (80-90%) were scraped using a sterile micropipette tip, and then serum-free medium was added. Next, cells were imaged at 0, 12 and 24 h using an inverted fluorescence microscope (magnification, x200; Olympus Corporation).

Transwell assay. In the migration test, the transfected cells ($1x10^5$) were inoculated into the top chamber (8 microns) with 200 μl serum-free medium. Complete medium (600 μl) containing 10% FBS was added to the lower chamber (Corning, Inc.). Following incubation at 37˚C for 24 h, the migratory cells located under the insert were fixed and stained with Crystal Violet Staining Solution (Beyotime Institute of Biotechnology) at room temperature for 30 min and observed using an inverted fluorescence microscope (magnification, x200; Olympus Corporation).

Statistical analysis. The χ^2 test was used to compare clinicopathological factors, and continuous variables were analyzed via unpaired Student's t-test or one-way ANOVA. Kaplan-Meier analysis and the log rank test were performed for survival analysis. Univariate and multivariate logistic regression models confirmed the associations between *DEFB4A* expression and clinical features. Prism 7 (GraphPad Software, Inc.) was used for statistical analysis of all clinical samples. ANOVA was followed by Tukey's post-hoc test and performed using SPSS 16.0 for Windows (SPSS, Inc.). R software (version 3.4; R Foundation for Statistical Computing) was used for bioinformatics analysis. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed in triplicate and data are presented as the mean ± standard deviation.

Results

Genes associated with poor prognosis. To identify genes associated with poor survival in the CRC cohort, patients were divided into two groups based on OS: Short (≦1,000 days) OS (patients with a survival time of 1,000 days would be included in short OS) and long (>1,000 days) OS (Fig. 1A). In total, 188 DEGs (fold change >2) were identified using a volcano plot, including 102 upregulated and 86 downregulated genes (Fig. 1B and C). Hierarchical cluster analysis revealed the expression profiles of the 188 DEGs (Fig. 1D). Subsequently, gene expression profiles in tumor and normal tissues (fold change >2) were analyzed, and it was observed that 916 genes were upregulated and 2,189 were downregulated in tumor tissues compared with in normal tissues ($P<0.05$, FDR <0.05, fold change >2; Fig. 1E-G). The Venn diagram revealed that

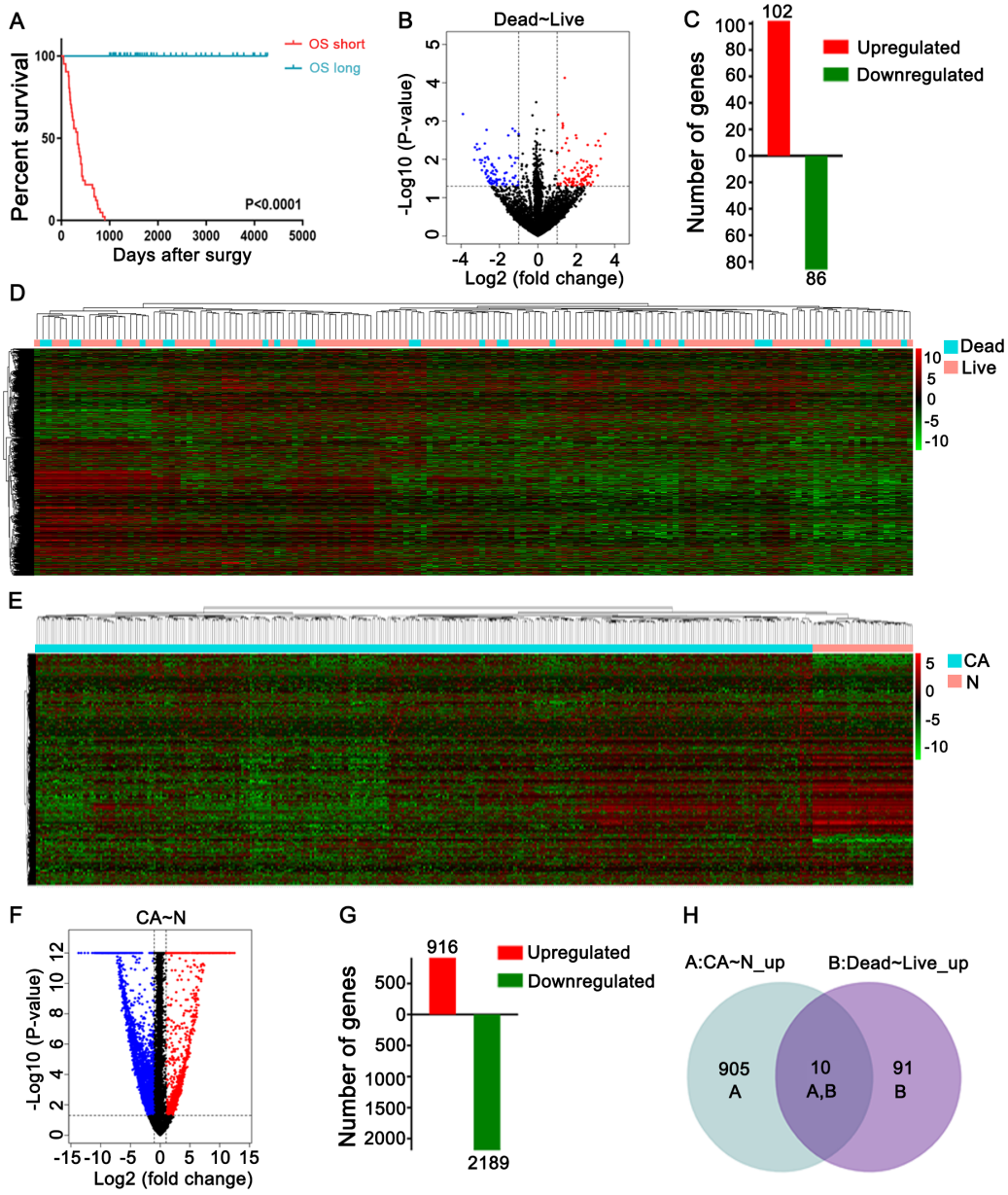

Figure 1. Screening of DEGs based on TCGA. (A) Patients with CRC were divided into two groups based on whether or not they survived for >1,000 days in accordance with the RNA-seq data from TCGA. (B) Volcano plot of RNA-seq data from TCGA. The red dots and blue dots represent upregulated and downregulated DEGs based on a fold change of >2. The volcano plot displays different genes when comparing patients with a prolonged OS and those with a short OS. (C) A total of 102 upregulated and 86 downregulated genes were identified. (D) Hierarchical clustering analysis of the RNA-seq data of different genes in short OS and long OS samples. (E) Hierarchical clustering analysis of the RNA-seq data of different genes in 689 CA and 95 N samples. (F) Using a threshold of P<0.05, false discovery rate <0.05 and fold change >2, DEGs were selected using a volcano plot when comparing 689 CA samples with 95 normal colon mucosa samples from TCGA. (G) A total of 916 upregulated and 2,189 downregulated genes were identified. (H) Venn diagram representing the distribution of DEGs in different groups. A total of 10 DEGs were expressed in both patients with CA and patients with a prolonged OS. CRC, colorectal carcinoma; DEGs, differentially expressed genes; OS, overall survival; TCGA, The Cancer Genome Atlas; CA, cancer; N, normal.

10 DEGs were identified in both screening methods (Fig. 1H). Details are shown in Table II.

Validated DEGs are associated with poor prognosis in TCGA. To determine the prognostic significance of the identified DEGs, their expression levels were determined in 784 cases included in TCGA. Kaplan-Meier survival analysis revealed that *DEFB4A*, hyaluronan binding protein 2 (*HABP2*), oleoyl-ACP hydrolase *(OLAH*) and TBC1 domain family member 3G (*TBC1D3G*) upregulation was significantly associated with poor survival in patients with CRC (Fig. 2A). Prognostic significance was not observed for *KISS1R*, *OR5M11*, *CHRNB3*, *OTX2*, *S100A7A*, *FLJ43860* and *FRMD7* in the patients with CRC (data not shown). Furthermore, *DEFB4A* and *HABP2* were upregulated in tumor tissues (Fig. 2B). A previous study have reported low serum expression levels of *HABP2* in patients with CRC (29). Therefore, *DEFB4A* was selected as a candidate marker of poor prognosis in patients with CRC.

GO and GSEA. To evaluate the biological role of *DEFB4A* in CRC progression, GO enrichment and GSEA analyses were

Table II. Upregulated genes (n=10) associated with a poor colorectal carcinoma prognosis in The Cancer Genome Atlas database.

Gene symbol	Gene ID	Description
DEFB4A	1673	Defensin beta 4A
HABP2	3026	Hyaluronan binding protein 2
OLAH	55301	Oleoyl-ACP hydrolase
TBC1D3G	101060321	TBC1 domain family member 3G
KISS1R	84634	KISS1 receptor
FRMD7	90167	FERM domain containing 7
S100A7A	338324	S100 calcium binding protein A7A
OTX2	5015	Orthodenticlehomeobox 2
OR5M11	219487	Olfactory receptor family 5 subfamily M member 11
CHRNB3	1142	Cholinergic receptor nicotinic beta 3 subunit

performed. *DEFB4A* was demonstrated to be involved in various biological processes (associated functional pathways are shown in Fig. 3A and B), and closely associated with 'myeloid leukocyte differentiation', 'leukocyte proliferation' and 'leukocyte mediated immunity', implying that DEFB4A potentially regulates the immune system. Finally, the database was searched for expression profiles of DEFB4A and immune-related genes, and a positive correlation between DEFB4A expression and the expression of immune markers, such as CD11b, CD14, CD45, CD163 and IL17A, was observed (Fig. 3C). These results suggest that DEFB4A is associated with poor prognosis in patients with CRC, potentially in an immunosuppressive myeloid leukocyte- and cytokine-dependent manner.

Validation in patient samples and clinical relevance of DEFB4A. To further clarify the clinical significance of *DEFB4A* expression, the present study analyzed tissue samples from 52 patients with CRC. The associations between their mRNA expression levels and clinicopathological variables were observed. Detailed information of the patients is provided in Table III. *DEFB4A* expression was significantly upregulated in the CRC tumor tissues (Fig. 4A). Additionally, an association between *DEFB4A* upregulation and advanced CRC stage (stage I, 12 cases; stage II, 16 cases; stage III, 16 cases; stage IV, 8 cases) and metastasis (M0, 44 cases; M1, 8 cases) was observed (Fig. 4B and C). Furthermore, *DEFB4A* upregulation in the tumor tissues was associated with poor prognosis (P=0.0313; Fig. 4D). Additionally, *DEFB4A* upregulation was significantly associated with advanced liver metastasis (P=0.039), stage (P=0.005), high CA72-4 value (P=0.003), tumor size (P=0.009) and lymph node metastasis (P=0.044; Table III). Therefore, *DEFB4A* was considered to be a prognostic marker associated with tumor progression in patients with CRC. Logistic regression analysis was performed to determine whether *DEFB4A* can help predict the prognosis of CRC. Univariate analyses revealed that advanced TNM stage [odds ratio (OR), 8.00; P=0.01], liver metastasis (OR, 4.21; P=0.03), lymph node metastasis (OR, 2.31; P=0.04), high CA199 level (OR,13.24; P=0.02), a high CA 72-4 level (OR, 10.19; P=0.01) and high DEFB4A level (OR, 2.15; P=0.02) were associated with the survival of patients with CRC. Furthermore, multivariate analyses revealed that advanced TNM stage (OR, 1.19; P=0.04), histological differentiation (OR, 0.67; P<0.01), liver metastasis (OR, 3.62; P=0.01), CA199 level (OR, 2.14; P=0.01), high CA 72-4 level (OR, 2.35; P=0.05) and high DEFB4A level (OR, 1.45; P=0.01) were independent prognostic predictors (Table IV). Overall, these results suggest that *DEFB4A* serves an important role in predicting the prognosis of patients with CRC.

DEFB4A promotes proliferation and metastasis in CRC. To explore the biological roles of DEFB4A in CRC, DEFB4A expression was knocked down in HCT116 and SW480 cells (Fig. 5A and B). A wound healing assay revealed that DEFB4A knockdown inhibited the migration of HCT116 and SW480 cells (Fig. 5C). Transwell assays demonstrated that the migration of cells was decreased following knockdown of DEFB4A in SW480 cells compared with that in the NC group (Fig. 5D). The number of migratory cells decreased following knockdown of DEFB4A in SW480 cells (Fig. 5E). Overall, these results suggested that DEFB4A serves an important role in CRC development.

Discussion

With the increasing availability of high-throughput technologies, numerous novel biomarkers and therapeutic targets have been identified through transcriptomic analysis of various types of tumor. However, such studies on biomarkers in CRC have not been extensively performed. The identification of CRC biomarkers may help predict and prolong the survival of patients with CRC.

In the present study, mRNA profiling of microarray analysis data from the TCGA database was performed to identify numerous novel genes associated with poor prognosis in CRC. A critical role of *DEFB4A* in patients with CRC was identified. The mRNA profiles of patients were first compared between the long OS and short OS groups, and between the tumor and normal tissue groups in the TCGA database. Subsequently, the present study investigated the association between mRNA expression and prognosis. *DEFB4A*, *HABP2*, *OLAH* and *TBC1D3G* were identified as potential predicators of poor prognosis. *DEFB4A* and *HABP2*

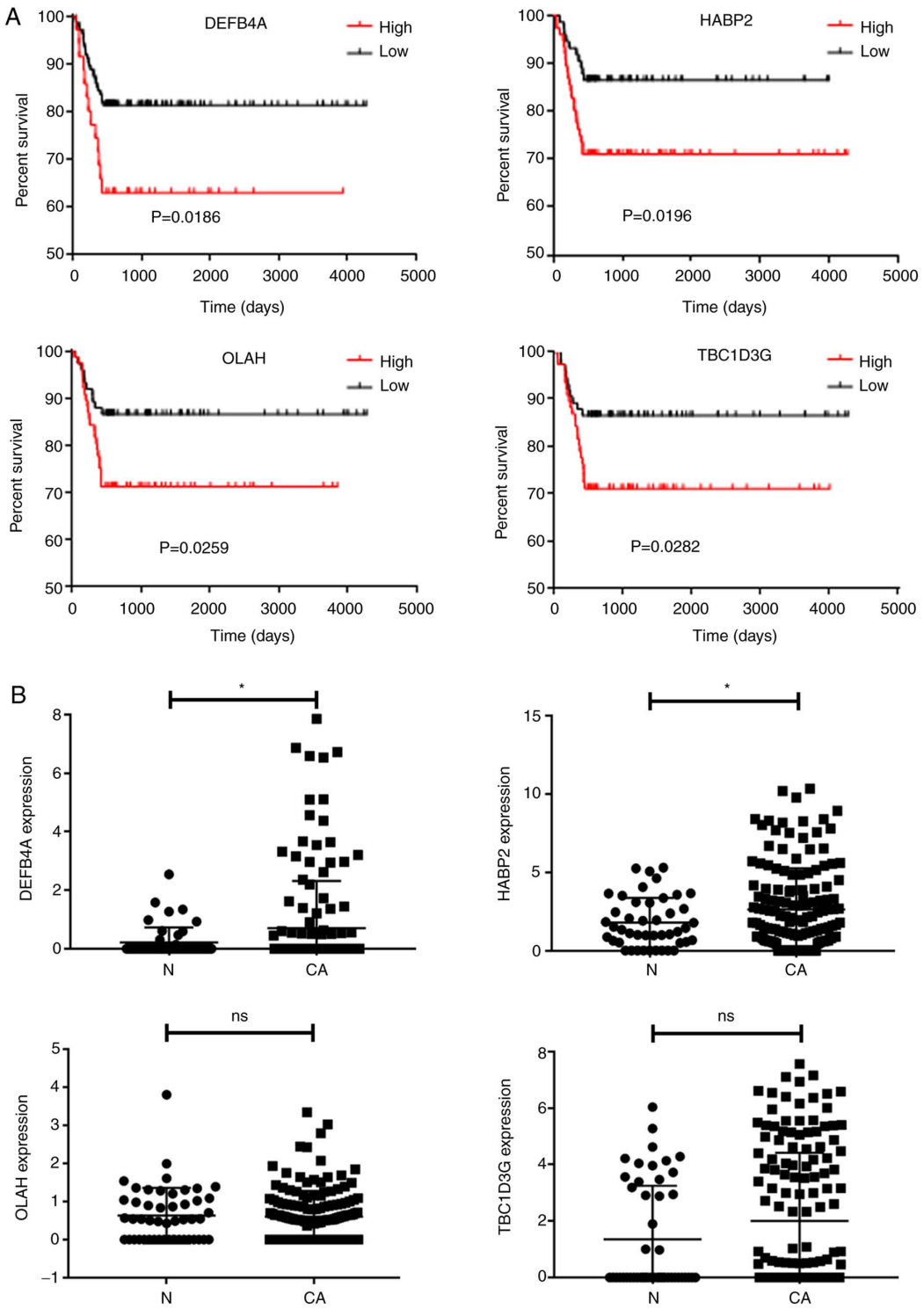

Figure 2. *DEFB4A* is upregulated based on data from TCGA and predicts poor prognosis. (A) Kaplan-Meier curve of four genes (*DEFB4A*, *HABP2*, *OLAH* and *TBC1D3G*) derived from data of patients included in the TCGA dataset. (B) mRNA expression levels of *DEFB4A*, *HABP2*, *OLAH* and *TBC1D3G* in cancer vs. control samples from patients in TCGA. *P<0.05. ns, not significant; TCGA, The Cancer Genome Atlas; DEFB4A, defensin β 4A; HABP2, hyaluronan binding protein 2; OLAH, oleoyl-ACP hydrolase; TBC1D3G, TBC1 domain family member 3G; CA, cancer; N, normal.

were upregulated in CRC tissues of patients in the database. However, *HABP2* has been reported to be downregulated in the sera of patients with CRC (P=0.0137) (29). Therefore, *DEFB4A* was considered as a candidate gene for further analysis. GO and GSEA were used to assess the function of *DEFB4A* in promoting disease progression and to highlight the role of *DEFB4A* in the tumor microenvironment. *DEFB4A* was involved in 'myeloid leukocyte differentiation', 'leukocyte proliferation' and 'leukocyte mediated immunity'. Correlation analysis revealed that *DEFB4A* expression was positively correlated with immune markers, including CD11b, CD14, CD45, CD163 and IL17A. CD11b is expressed on the surface of a number of leukocytes, including monocytes, granulocytes and macrophages (30). CD14 is expressed on both monocytes

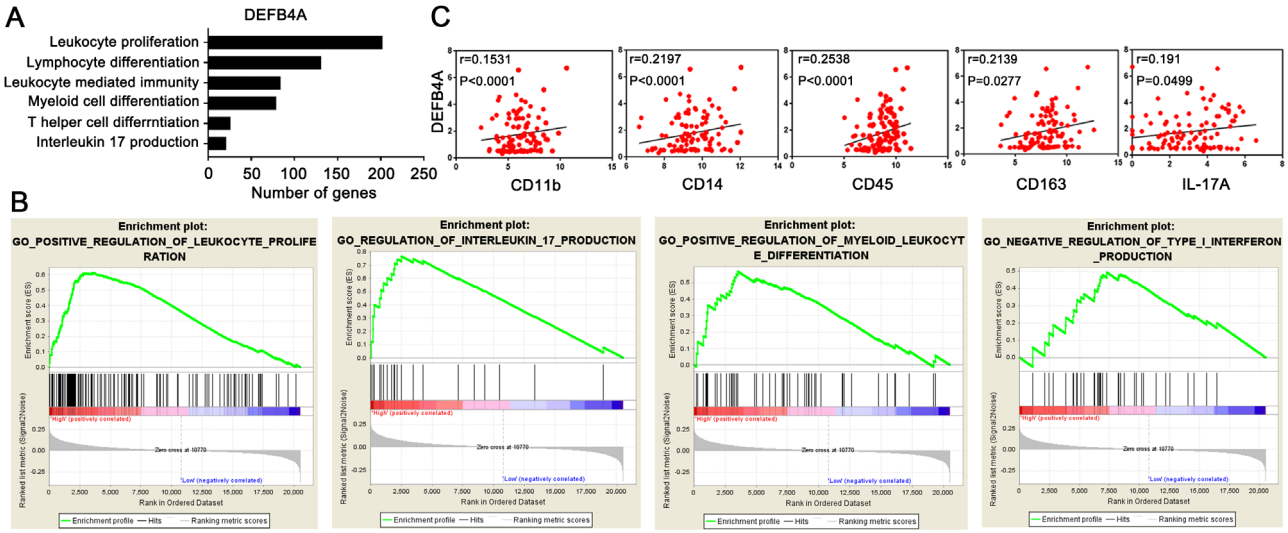

Figure 3. *DEFB4A* is positively correlated with inhibitory immune cells. (A) Gene Ontology analysis revealed that *DEFB4A* is involved in 'leukocyte proliferation', 'lymphocyte differentiation', 'leukocyte mediated immunity', 'myeloid cell differentiation', 'negative regulation of type I interferon production' and 'interleukin-17 production'. (B) Gene set enrichment analysis verified the results. (C) Correlation between *DEFB4A* and CD11b, CD14, CD45, CD163 and IL17A. r and P-values are indicated. DEFB4A, defensin β 4A; r, Pearson's correlation coefficient.

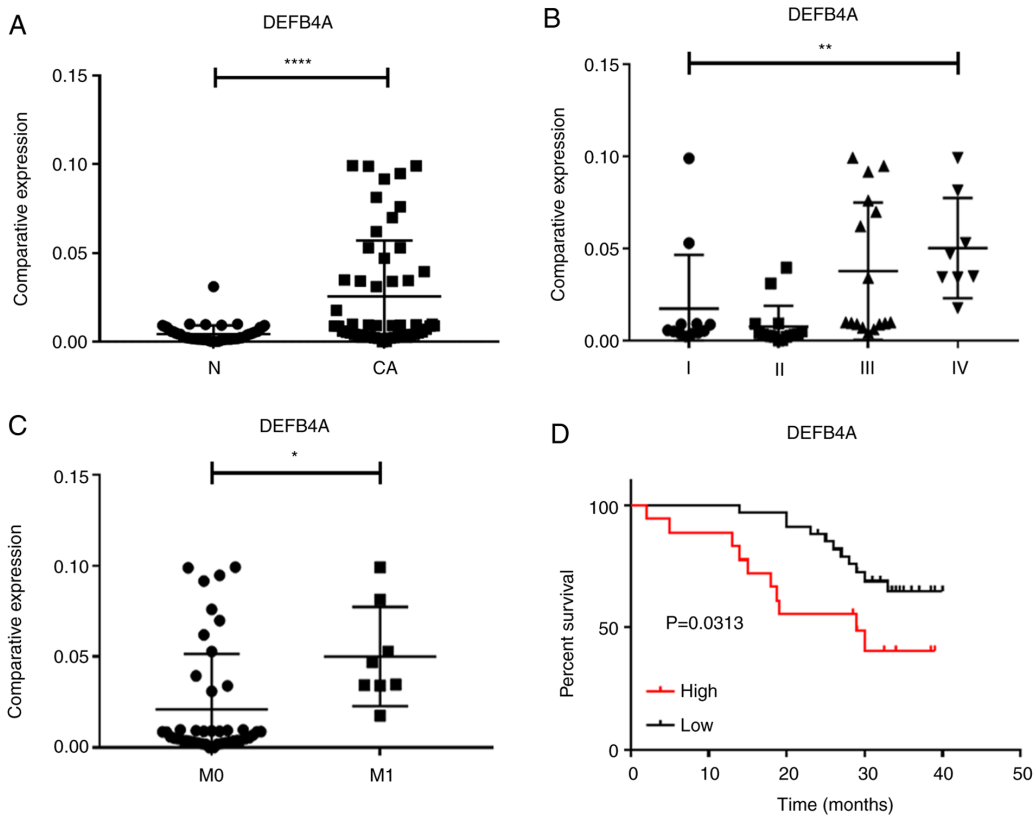

Figure 4. *DEFB4A* predicts poor prognosis in colorectal cancer. (A) DEFB4A mRNA expression in groups of cancer tissues and normal tissues are displayed. (B) DEFB4A mRNA levels were compared with respect to TNM stage. (C) DEFB4A mRNA expression levels of patients with different cancer stages. (D) Effect of DEFB4A expression on overall survival in patients with colorectal carcinoma (n=52). *P<0.05; **P<0.01; ****P<0.0001. DEFB4A, defensin β 4A.

and macrophages, and CD45 is expressed on leukocytes. M2 macrophages may be marked with CD163 and M2 macrophages serve a role in promoting tumor growth (31). The OS of patients with non-small-cell lung cancer (32) and those with esophageal cancer (33,34), with high M2 macrophage infiltration rates is shorter than those with low M2 macrophage infiltration rates. Patients with high expression levels of IL-17A had a poor prognosis in a CRC cohort (35). Previous studies have suggested that increased IL-17A promotes CRC in various animal models (36-38).

Analysis of clinical specimens of patients with CRC demonstrated that *DEFB4A* expression was associated with

Table III. Association between DEFB4A expression and clinicopathological characteristics of patients with colorectal carcinoma.

		DEFB4A expression			
Characteristic	Total, n	High, n	Low, n	χ^2	P-value
Sex				0.000	>0.999
Male	34	17	17		
Female	18	9	9		
Age, years				0.000	>0.999
<60	26	11	15		
≥60	26	15	11		
Site of lesion				0.077	0.785
Colon	21	13	8		
Rectum	31	13	18		
Differentiation				1.194	0.330[a]
Poor	12	3	9		
Well	40	23	17		
Tumor size, cm				7.212	0.009[a]
<5	28	21	7		
≥5	24	20	4		
Pathological type				1.000	0.575[a]
Adenocarcinoma	47	25	22		
Others	5	3	2		
Lymph node metastasis				4.064	0.044
No	33	20	13		
Yes	19	13	6		
Liver metastasis				5.005	0.039[a]
No	44	26	18		
Yes	8	8	0		
Stage				8.026	0.005
I/II	28	18	10		
III/IV	24	18	6		
CEA				0.001	0.974
Normal	29	15	14		
High	23	11	12		
CA 19-9				3.315	0.139
Normal	42	25	17		
High	10	1	9		
CA 72-4				5.678	0.003[a]
Normal	38	14	24		
High	14	2	12		

[a]Fisher's exact test. All others were assessed using a χ^2 test. DEFB4A, defensin β 4A.

poor survival. Furthermore, *DEFB4A* expression was upregulated in patients with CRC with advanced and metastatic cancer. Patients with CRC with high *DEFB4A* expression had poor survival. In addition, knockdown of DEFB4A affected the migration ability of CRC cells.

TCGA data of patients with CRC were used to identify the DEGs between the long OS (>1,000 days) and short OS (<1,000 days) groups. In addition, mRNA expression was compared between tumor tissues and normal tissues in the same database. DEFB4A was highly expressed in tumors and associated with a poor prognosis. DEFB4A upregulation was associated with poor prognosis, and DEFB4A expression was significantly upregulated in patients with large tumors, advanced cancer stage, lymph node metastasis and liver metastasis. Another study used the Gene Expression Omnibus database to screen genes that are increased in patients with recurrence (39). Hierarchical clustering and pathway analyses revealed that thrombospondin 2 (THBS2) and cartilage

Table IV. Logistic regression model analysis of liver metastasis predictors in patients with colorectal carcinoma.

	Univariate			Multivariate		
Characteristics	OR	95% CI	P-value	OR	95% CI	P-value
Sex (male vs. female)	1.00	0.32-3.14	>0.99	0.78	0.06-10.56	0.85
Age (<60 vs. ≥60 years)	0.54	0.18-1.62	0.27	13.05	0.93-183.71	0.06
Tumor size (<50 vs. ≥50 mm)	1.00	0.34-2.98	>0.99	0.00	0.09-9.13	>0.99
Pathological type (adenocarcinoma vs. others)	3.57	0.75-10.28	0.06	2.45	0.61-2.76	0.32
TNM stage (I/II vs. III/IV)	8.00	2.42-16.81	0.01	1.19	1.04-2.30	0.04
Differentiation (medium vs. poor)	0.34	0.10-1.18	0.09	0.67	12.34-20.79	<0.01
Liver metastasis (no vs. yes)	4.21	1.35-8.42	0.03	3.62	1.24-7.78	0.01
Lymph node metastasis (no vs. yes)	2.31	1.24-3.56	0.04	24.86	0.31-35.96	0.92
CEA (<5 vs. ≥5)	1.17	0.39-3.50	0.78	0.32	0.08-3.02	0.78
CA199 (<35 vs. ≥35)	13.24	1.53-114.30	0.02	2.14	1.03-3.72	0.01
CA724 (<6.9 vs. ≥6.9)	10.19	2.00-52.80	0.01	2.35	2.00-5.80	0.05
DEFB4A (high vs. low)	2.15	1.43-2.86	0.02	1.45	1.02-1.89	0.01

OR, odds ratio.

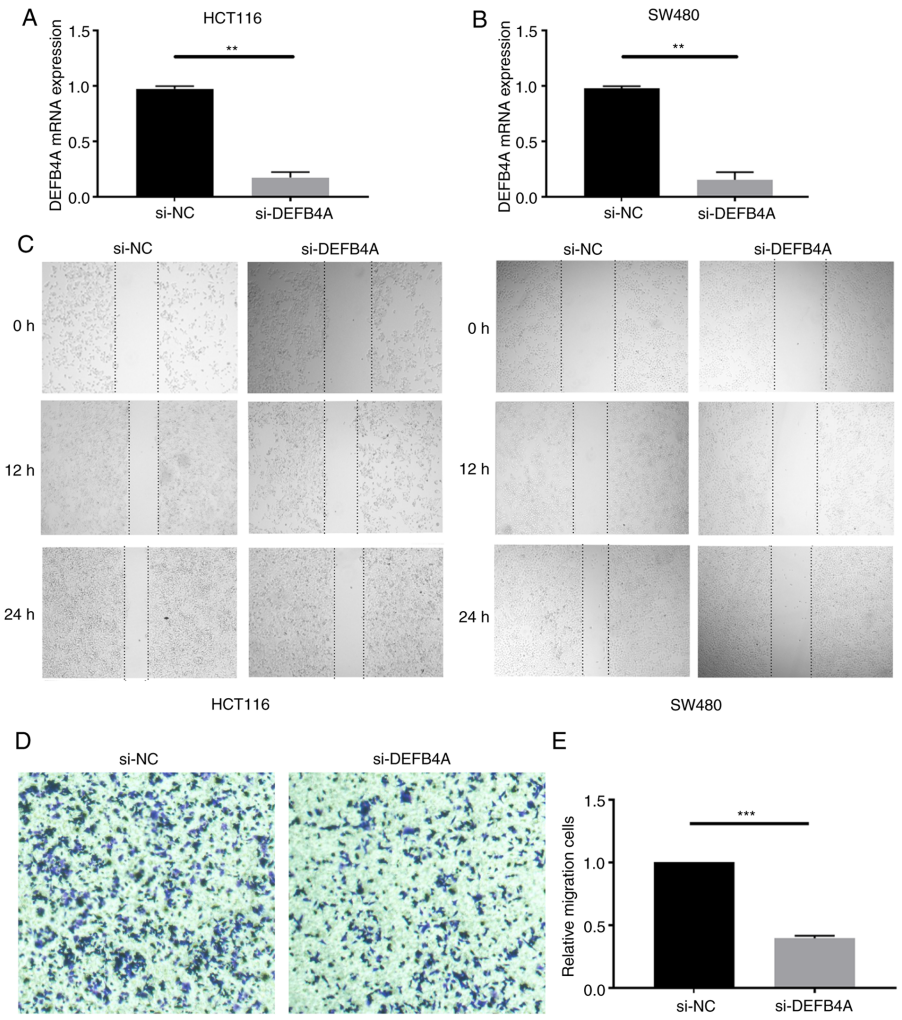

Figure 5. DEFB4A promotes colorectal cancer cell migration. (A) Expression levels of DEFB4A were detected using RT-qPCR following transfection of HCT116 cells with si-NC and si-DEFB4A. (B) Expression levels of DEFB4A were detected using RT-qPCR following transfection of SW480 cells with si-NC and si-DEFB4A. (C) Migration ability of cells was examined using a wounding healing assay. (D) Representative images were obtained for the Transwell assay (magnification, x200). (E) Proportions of migrated cells after 24 h were quantified. DEFB4A, defensin β 4A; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering RNA. **P<0.01; ***P<0.001.

oligomeric matrix protein (COMP) are associated with the ECM-receptor interaction, focal adhesion and TGF-β signaling pathways (39). The hypergeometric distribution test demonstrated that the association between THBS2 and CRC is stronger than that of COMP (39). Pearson test results indicated that THBS2 might be considered to be a prognostic biomarker for CRC (39). To the best of our knowledge, this screening method and the hypothesis that DEFB4A may serve a pro-tumor role through immunosuppression have not been seen in other studies.

DEFB4A stimulates keratinocytes to release IL-18 and IL-20, pro-inflammatory cytokines serving as deciding factors in the pathogenesis of psoriasis (40). Furthermore, *DEFB4A* induction is required for Toll-like receptor (TLR) activation in monocytes through the convergence of IL-1 and vitamin D receptor signaling, and exerts direct bactericidal effects against *M. tuberculosiss* (41). The antimicrobial peptides *DEFB4A* and CAMP are inhibited by hsa-miR-21, leading to suppression of the TLR2/1-induced vitamin D antimicrobial signaling pathway (42). *DEFB4A* has been suggested as a biomarker for psoriasis because the clinical efficacy of targeted antibody therapy in psoriasis is associated with the inhibition of *DEFB4A* expression (43). *DEFB4A* expression can directly be inhibited by anthralin *in vitro* and *in vivo*, thus benefiting patients with psoriasis (44). However, it has remained unclear whether *DEFB4A* is involved in the immunoregulation in CRC. GO analysis revealed that *DEFB4A* is involved in 'myeloid leukocyte differentiation', 'leukocyte proliferation' and 'positive regulation of leukocyte-mediated immunity'. Therefore, *DEFB4A* may be associated with immunity in CRC.

To the best of our knowledge, the present study was the first to report *DEFB4A* as a prognostic marker for CRC and as an immunoregulatory factor in the tumor microenvironment in patients with CRC. However, a limitation of the present study was that the research cohort was not large enough, which may affect the statistical results. In addition, the specific role of DEFB4A and immune factors in colon cancer and the underlying molecular mechanism need to be further explored.

In conclusion, to the best of our knowledge, *DEFB4A* is upregulated in patients with CRC and is closely associated with poor prognosis. *DEFB4A* regulates immune function and potentially promotes immunosuppression. Therefore, *DEFB4A* may be considered as a prognostic marker and immunotherapeutic target for CRC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YZ, QW and DW participated in the design and conception of the present study. YZ, QW, DW, ZS, JL and WTY were involved in data acquisition and analysis of certain clinical data. QW, DW, ZZ and YW performed the clinical experiments and analysis of the data. The manuscript was written by QW and critically reviewed by YZ, DW, ZZ, YW, WNY and NRM. WNY, KS and NRM were involved in performing and analyzing the cell experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Zhengzhou University (approval no. Science-2010-LW-1213), and informed consent was obtained from each patient with available follow-up information.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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