DEFB4A is a potential prognostic biomarker for colorectal cancer

QIAN WU1,2*, DAN WANG1,2*, ZHEN ZHANG1,2, YAPING WANG1,2, WEINA YU1,2, KAI SUN1,2, NOMATHAMSANQA RESEGOSETSE MAIMELA1,2, ZHENQIANG SUN3, JINBO LIU, WEITANG YUAN3 and YI ZHANG1,2,4,5

1Biotherapy Center; 2Cancer Center; 3Department of Anorectal Surgery, The First Affiliated Hospital of Zhengzhou University; 4School of Life Sciences, Zhengzhou University; 5Henan Key Laboratory for Tumor Immunology and Biotherapy, Zhengzhou University, Zhengzhou, Henan 450052, P.R. China

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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 DEFB4A 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.

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

*Contributed equally

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Correspondence to: Dr Yi Zhang, Biotherapy Center, The First Affiliated Hospital of Zhengzhou University, 1 Jianshe East Road, Zhengzhou, Henan 450052, P.R. China
E-mail: yizhang@zzu.edu.cn
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.ssgdb.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 CTCTGTCAGTCCTCTTCTCT-3' and reverse, 5'-CGACGAA AACAGGATCGCCTAT-3'; and GAPDH forward, 5'-GGA GCGAGATCCCTCCAAAT-3' and reverse, 5'-GGCTGTGTT
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% CO₂. 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'-UUCUCUUCAUAUUCC UGATT-3' and antisense: 5'-AUCAGGAUAUGAGG 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% CO₂ 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 $\chi^2$ 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
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 has 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
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.

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 predictors of poor prognosis. DEFB4A and HABP2

| Gene symbol | Gene ID | Description |
|-------------|---------|-------------|
| DEFB4A      | 1673    | Defensin beta 4A |
| HABP2       | 3026    | Hyaluronan binding protein 2 |
| OLAH        | 55301   | Oleyl-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 |
| CHRNA3      | 1142    | Cholinergic receptor nicotinic beta 3 subunit |

Table II. Upregulated genes (n=10) associated with a poor colorectal carcinoma prognosis in The Cancer Genome Atlas database.
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 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 β4A; HABP2, hyaluronan binding protein 2; OLAH, oleyl-ACP hydrolase; TBC1D3G, TBC1 domain family member 3G; CA, cancer; N, normal.
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 \textit{DEFB4A} expression was associated with
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

| Characteristic                      | Total, n | High, n | Low, n | χ²  | P-value |
|-------------------------------------|----------|---------|--------|-----|---------|
| Sex                                 |          |         |        |     |         |
| Male                                | 34       | 17      | 17     | 0.000 | >0.999  |
| Female                              | 18       | 9       | 9      | 0.000 | >0.999  |
| Age, years                          |          |         |        |     |         |
| <60                                 | 26       | 11      | 15     | 0.077 | 0.785   |
| ≥60                                 | 26       | 15      | 11     | 0.077 | 0.785   |
| Site of lesion                      |          |         |        |     |         |
| Colon                               | 21       | 13      | 8      | 1.194 | 0.330<sup>a</sup> |
| Rectum                              | 31       | 13      | 18     | 1.194 | 0.330<sup>a</sup> |
| Differentiation                     |          |         |        |     |         |
| Poor                                | 12       | 3       | 9      | 7.212 | 0.009<sup>a</sup> |
| Well                                | 40       | 23      | 17     | 7.212 | 0.009<sup>a</sup> |
| Tumor size, cm                      |          |         |        |     |         |
| <5                                  | 28       | 21      | 7      | 4.064 | 0.044   |
| ≥5                                  | 24       | 20      | 4      | 4.064 | 0.044   |
| Pathological type                   |          |         |        |     |         |
| Adenocarcinoma                      | 47       | 25      | 22     | 1.000 | 0.575<sup>a</sup> |
| Others                              | 5        | 3       | 2      | 1.000 | 0.575<sup>a</sup> |
| Lymph node metastasis               |          |         |        |     |         |
| No                                  | 33       | 20      | 13     | 5.005 | 0.039<sup>a</sup> |
| Yes                                 | 19       | 13      | 6      | 5.005 | 0.039<sup>a</sup> |
| Liver metastasis                    |          |         |        |     |         |
| No                                  | 44       | 26      | 18     | 8.026 | 0.005   |
| Yes                                 | 8        | 8       | 0      | 8.026 | 0.005   |
| Stage                               |          |         |        |     |         |
| I/II                                | 28       | 18      | 10     | 0.001 | 0.974   |
| III/IV                              | 24       | 18      | 6      | 3.315 | 0.139   |
| CEA                                 |          |         |        |     |         |
| Normal                              | 29       | 15      | 14     | 5.678 | 0.003<sup>a</sup> |
| High                                | 23       | 11      | 12     | 5.678 | 0.003<sup>a</sup> |
| CA 19-9                             |          |         |        |     |         |
| Normal                              | 42       | 25      | 17     | 3.315 | 0.139   |
| High                                | 10       | 1       | 9      | 3.315 | 0.139   |
| CA 72-4                             |          |         |        |     |         |
| Normal                              | 38       | 14      | 24     | 5.678 | 0.003<sup>a</sup> |
| High                                | 14       | 2       | 12     | 5.678 | 0.003<sup>a</sup> |

<sup>a</sup>Fisher's exact test. All others were assessed using a χ² test. DEFB4A, defensin β4A.
Table IV. Logistic regression model analysis of liver metastasis predictors in patients with colorectal carcinoma.

| Characteristics                                      | Univariate                  |          |          | Multivariate |          |          |
|-------------------------------------------------------|----------------------------|----------|----------|--------------|----------|----------|
|                                                        | 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 β4A; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering RNA. **P<0.01; ***P<0.001.
olfemocytic 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. tuberculosis (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 antrhalin 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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