Low Expression of AGPAT5 Is Associated With Clinical Stage and Poor Prognosis in Colorectal Cancer and Contributes to Tumour Progression

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ABSTRACT

BACKGROUND: Colorectal cancer (CRC) has a high prevalence and poor prognosis. This study aimed to identify biomarkers related to the clinical stage (I-IV) of CRC.

METHODS: The LinkedOmics database was used as the discovery cohort, and two Gene Expression Omnibus (GEO) databases (GSE41258 and GSE422848) served as validation cohorts. The trend test of genes related to clinical stage (I-IV) of CRC patients was identified by the Jonckheere-Terpstra test. The cBioPortal database, Gene Expression Profiling Interactive Analysis (GEPIA) and Prognoscan databases were used to explore the expression change and prognostic value of clinical stage-related genes in CRC patients. CRC cells overexpressed AGPAT5 were constructed and used for cell counting kit-8 (CCK-8), flow cytometric, and wound healing assays in vitro.

RESULTS: We identified four clinical stage-related genes, GSR, AGPAT5, CRLF1, and NPR3, in CRC. The CNA frequencies of GSR, CRLF1, AGPAT5, and NPR3 occurred in 11%, 2.4%, 13%, and 3% of patients, respectively. The expression of GSR and AGPAT5 tended to decrease with CRC stage (I-IV) progression, and the expression of CRLF1 and NPR3 tended to increase with CRC stage (I-IV) progression. Compared with the normal group, AGPAT5 expression was markedly decreased in stage IV CRC. Higher GSR and AGPAT5 expression levels were associated with better overall survival (OS) and disease-free survival (DFS) in CRC patients. Lower CRLF1 and NPR3 expression levels were associated with better OS and DFS in CRC. GSR, CRLF1, AGPAT5, and NPR3 expression were related to CRC progression, microsatellite instability, and tumour purity in CRC. Furthermore, AGPAT5 was downregulated in CRC cell lines, and overexpression of AGPAT5 inhibited cell proliferation and migration and promoted cell apoptosis in CRC cells.

CONCLUSION: Low AGPAT5 expression may serve as a poor prognostic factor and clinical stage biomarker in CRC. In addition, AGPAT5 acts as a tumour suppressor in CRC progression.

KEYWORDS: Colorectal cancer, clinical stage, prognosis, biomarkers, AGPAT5, tumour suppressor

Introduction

Colorectal cancer (CRC) is one of the most malignant tumours of the digestive system and was the second most common cause of cancer-related mortality in 2018.1,2 Recently, the overall survival (OS) of CRC has been improved through treatments, such as surgery, radiotherapy, chemotherapy, immunotherapy, and targeted therapy.3 A statistical analysis reported that the 5-year survival rate of patients was more than 90% for stage I CRC, and approximately, 10% for stage IV CRC.2 The survival rate of patients with metastasized CRC is still poor.4,5 Aberrant genomic expression plays a crucial role in CRC development. Therefore, it is very important to identify novel clinical stage-related biomarkers for the treatment and diagnosis of CRC.

Bioinformatics, one of the new disciplines based on the intersection of molecular biology and many disciplines, has been widely used to explore the changes and significance of the genome in tumours.6 The expression and function of hub genes have been reported in CRC pathogenesis and as biomarkers for targeted therapy in CRC.6,7 For example, 10 key hub genes were associated with CRC, in which HCAR3 and INSL5 were identified and verified as new potential therapeutic targets in CRC.8 Foetal-lethal noncoding developmental regulatory RNA (FENDRR) has been reported as a potential biomarker for the diagnosis and prognosis of CRC.9 However, these biomarkers have not been identified for their potential clinical significance in CRC.

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The Jonckheere-Terpstra (JT) test is a nonparametric method used to test whether there are significant differences in the distribution of multiple populations from multiple independent samples.10 The JT test is a differential analysis of disease progression markers.11 CRC has four stages of development: I, II, III, and IV. The JT test was used to analyse whether gene expression increased or decreased progressively with cancer progression. With the increase of colon cancer incidence and mortality trends and unpredictability of factors contributing to local tumour metastatic potential, we urgently need to identify the genes responsible for colon cancer progression. Increased understanding of the genes involved in tumour stage may be useful for early diagnosis and for identifying targets for personalized cancer treatment.

The 1-acylglycerol-3-phosphate O-acyltransferases (AGPATs) are a family of enzymes that catalyse the synthesis of phosphatidic acid (PA), an intermediate in triacylglycerol (TAG) synthesis, a signalling molecule, and a precursor of phospholipids. The AGPAT family consists of five membrane-bound acyltransferases (AGPAT1-5). Importantly, the expression of AGPATs has been linked to diverse physiological and pathological phenotypes, including cancer.12 AGPATs influence cancer cell survival and proliferation.13 AGPAT2 was upregulated in various cancers, and its specific inhibition has been investigated as a potential targeted cancer therapy.14,15 Previous studies found that AGPAT4/lysophosphatidic acid (LPA) in CRC cells regulated antitumour immunity via p38/ERK1/2, M1/M2 macrophages, and JAK/STAT3 activation.16 AGPAT1 was known to contribute to the lipid metabolism signature and predict prognosis from GSE41258 and GSE42284 by the JT test. The expression profiles of GSR, CRLF1, AGPAT5, and NPR3 in the normal group and the stage I to IV groups were analysed by t-test. The log2 fold changes of GSR, CRLF1, AGPAT5, and NPR3 between the normal and stage I to IV groups were analysed. Red represents upregulation and blue represents downregulation.

Materials and Methods

**LinkedOmics database analysis**

The LinkedOmics database (http://www.linkedomics.org/admin.php) is a web portal that analyses multiomics data from The Cancer Genome Atlas (TCGA) data sets.18 We selected the TCGA colorectal adenocarcinoma (COADREAD) sample to search for an RNA-sequence data set related to the clinicopathological stage using the LinkFinder module. The clinical stage-related genes were screened out by the JT test based on CRC clinical information from 629 patients and RNA-seq data from 379 patients. The clinicopathological stage-related genes were visualized in a volcano plot and a heatmap. We set thresholds of |rank correlation (JT trend test)| > 0.2 and -log10 (P value) > 1.3.

**Validation of clinical stage-related gene expression trends**

To validate the clinical stage-related gene expression in clinical stage (I-IV), two independent data sets (GSE4125819 and GSE4228420) were downloaded from the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) database. All gene expression profile matrices were standardized by R software, and we further analysed the gene expression in terms of clinical stage (I-IV). The clinical stage-related gene expression was further validated in CRC stage (I-IV) progression from GSE41258 and GSE42284 by the JT test. The expression profiles of GSR, CRLF1, AGPAT5, and NPR3 in the normal group and the stage I to IV groups were analysed by t-test. The log2 fold changes of GSR, CRLF1, AGPAT5, and NPR3 between the normal and stage I to IV groups were analysed. Red represents upregulation and blue represents downregulation.

**Analysis of copy number alteration**

The copy number alteration (CNA) was analysed by the cBioPortal database. We searched the cBioPortal for Cancer Genomics (http://cbioportal.org) database to explore the alteration frequencies of GSR, CRLF1, AGPAT5, and NPR3 in CRC patients.

**Prognostic value analysis of clinical stage-related genes**

The Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia2.cancer-pku.cn/#analysis) and PrognoScan database (http://dna00.bio.kyutech.ac.jp/PrognoScan/index.html) were used to explore the prognostic value of clinical stage-related genes in CRC patients. The GEPIA database is an online website that analyses the correlation between clinical stage-related gene expression and OS and disease-free survival (DFS) in CRC. In the GEPIA database, the median gene expression was used as a cut-off value to classify groups. Data on the PrognoScan website were also collected from the GEO and TCGA databases. The prognostic value was considered statistically significant when the P value was less than 0.05.

**Acquisition of gene expression and clinical information**

The gene expression profile matrix and clinical information of CRC were downloaded from TCGA (https://tcga-data.nci.nih.gov/tcga/). All gene expression profile matrices were standardized by R software, and we further overlapped GSR, CRLF1, AGPAT5, and NPR3 mRNA to obtain GSR, CRLF1, AGPAT5, and NPR3 expression, and clinical information. GSR, CRLF1, AGPAT5, and NPR3 expression data in the N,
T, and M stages, and microsatellite instability (MSI) statuses were visualized by GraphPad software. Correlations of GSR, CRLF1, AGPAT5, and NPR3 expression with tumour purity (TP) were evaluated by the Pearson correlation coefficient.

Cell culture

The human normal epithelial cell line (HIEC) and CRC cell lines (HCT116 and SW480) were purchased from the American-Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640, Gibco, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibco, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin (Gibco, USA) and maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Construction of pcDNA3.1-AGPAT5 and cell transfection

The overexpression plasmid pcDNA3.1-AGPAT5 and the control vector pcDNA3.1 were purchased from GenePharma (Shanghai, China). For cell transfection, CRC cell lines (HCT116 and SW480) were transfected with pcDNA3.1-AGPAT5 or empty vector using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer’s instructions.

Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted from cells using TRIzol (Invitrogen, USA). cDNA was synthesized using a reverse transcription kit according to the manufacturer’s instructions. The cDNA was amplified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using AceQ Universal SYBR qPCR Master Mix on an ABI 7500 PCR system and the following conditions: (a) initial denaturation at 95°C for 5 min, (b) 40 cycles of 95°C for 10 s and 60°C for 30 s, and (c) 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. Data analyses for gene expression were performed using the 2^ΔΔCt method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences were as follows: GAPDH-F: 5'-GAAGGTGAAGGTCGGAGTC-3', GAPDH-R: 5'-GAAGATGGTGATGGGATTT-3', AGPAT5-F: CTGTGCCAGCACACGTACTC, AGPAT5-R: 5'-CCAGGCCCCACACGTAGTG-3'.

Cell proliferation assay

Cell proliferation was analysed by a cell counting kit-8 (CCK-8, Dojindo, Japan) assay after treatment. In brief, logarithmic growth cells (1 × 10⁵) were seeded into 96-well cell culture plates with four replicate wells for each treatment. At 0, 24, 48, and 72h, 10 μL of CCK-8 solution was added to each well. The cells were incubated in a cell incubator for 1 h. The absorbance of each well was determined by enzyme label detection at 450 nm (Molecular Devices, USA). The experiment was performed three times in triplicate.

Flow cytometric assay

Cell apoptosis was assessed using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences, USA) according to the manufacturer’s instructions. In brief, HCT116 and SW480 cells were collected and washed with PBS after relevant treatment and transfection. Then, 500 μL binding buffer was used to resuspend the cells, and 5 μL Annexin V-FITC and 5 μL PI were used to stain the cells at room temperature in the dark for 15 min. Finally, the cell apoptotic rate was analysed by a FACScan® flow cytometer (BD Biosciences) using FlowJo software (7.6.1, FlowJo LLC).

Cell migration assay

Cell migration was analysed by wound healing assay. In brief, CRC cells were seeded in six-well cell culture plates for each treatment. When the cells had grown to 100% confluency, the tip of a 200-μL pipette was used to make a scratch on the monolayer. PBS was applied to wash off the scratched cells three times, and the cells were supplemented with serum-free medium. Cell migration was monitored under a microscope and then photographed at 0 and 48 h. The experiment was performed three times in triplicate.

Statistical analysis

The data collected from three independent experiments are expressed as the mean ± standard deviation (SD). GraphPad Prism 8.0 was used to perform the statistical analyses. Paired Student’s t-test or one-way analysis of variance (ANOVA) was used for analysis with multiple comparisons. Kaplan-Meier analyses and log-rank tests were used to evaluate the relationships between groups, and OS and DFS. Correlations between continuous variables were evaluated by Pearson’s correlation coefficients. P < 0.05 was considered statistically significant.

Results

Identification of clinical stage-related genes in TCGA COADREAD

To explore the biomarkers related to the clinical stage in CRC, the LinkedOmics database was used as the discovery cohort, and two GEO databases (GSE41258 and GSE422848) served as validation cohorts. The trend of genes related to clinical stage (I-IV) of CRC patients was identified by the JT test. The volcano plot showed that the 50 genes tended to increase with CRC stage (I-IV) progression, and 50 genes tended to decrease...
with CRC stage (I-IV) based on the thresholds -log10(P value) > 1.3 and |rank correlation (JT trend test)| > 0.2 (Figure 1A). The heatmap showed the expression levels of 50 upregulated genes in CRC patients (stages I-IV), including CRLF1 and NPR3 (Figure 1B). Next, the expression levels of 50 downregulated genes in CRC patients (stages I-IV), including GSR and AGPAT5, were analysed.

Validation of clinical stage-related genes in GSE41258 and GSE42284

To further validate the expression trend of clinical stage-related genes in CRC stage (I-IV), we downloaded the GSE41258 and GSE42284 data sets from the GEO data set. A heatmap was used to display the expression profiles of the 53 genes significantly related to clinical stage (I-IV) based on GSE41258 (Figure 2A). Twelve common clinical stage-related genes were identified, including GSR, CRLF1, AGPAT5, and NPR3 (Figure 2B). Next, the heatmap showed that 62 genes significantly related to clinical stage were identified in clinical stage (I-IV) based on the GSE42284 data set (Figure 2C). Sixteen common clinical stage-related genes were identified, including GSR, CRLF1, AGPAT5, and NPR3 (Figure 2D).

CNA and expression of GSR, CRLF1, AGPAT5, and NPR3 in CRC

Venn diagrams identified four common clinical stage-related genes, including GSR, CRLF1, AGPAT5, and NPR3 (Figure 3A). Next, the CNAs of GSR, CRLF1, AGPAT5, and NPR3 were analysed using the cBioPortal website. The results showed that genomic alterations of GSR, CRLF1, AGPAT5, and NPR3 occurred in 11%, 2.4%, 13%, and 3% of patients, respectively (Figure 3B). The expression profiles of GSR, CRLF1, AGPAT5, and NPR3 in clinical stage (I-IV) were analysed in the GSE41258 and GSE42284 data sets by the JT trend test. As shown in Figure 3C and D, the expression trends of GSR and AGPAT5 were significantly decreased with cancer stage progression, and the expression trends of CRLF1 and NPR3 were significantly increased with cancer progression in CRC patients. Furthermore, the expression levels of GSR, CRLF1, AGPAT5, and NPR3 in the normal and stage I to IV groups were analysed. As shown in Figure 3E, compared with the normal group, the expression of GSR was decreased in stages III and IV of CRC, and the expression of AGPAT5 was reduced in stage IV of CRC. Moreover, the fold changes in GSR, CRLF1, AGPAT5, and NPR3 expression between the normal group and the stage I to IV groups were presented in a heatmap. Compared with the normal, and stage I to III groups, GSR and AGPAT5 expression levels were reduced in stage IV. Compared with the normal group, the trends of GSR and AGPAT expression were reduced in stages I to IV, and the trends of CRLF1 and NPR3 expression were increased in stages I to IV (Figure 3F). The trends of gene expression related to stage were consistent with the results of the JT trend test.

Prognostic value analysis of clinical stage-related genes

The prognostic value of GSR, CRLF1, AGPAT5, and NPR3 expression in CRC patients was analysed by several databases. In GEPIA, we found that lower GSR and AGPAT5 expression were associated with poorer OS, and higher CRLF1 expression was associated with poorer OS and DFS in CRC patients (Figure 4A). On the PrognoScan website, we found that lower GSR and AGPAT5 expression levels were associated with poorer DFS, and higher NPR3 expression was associated with poorer OS and DFS in CRC patients (Figure 4B). These results revealed that GSR, CRLF1, AGPAT5, and NPR3 expression levels were closely related to the cancer prognosis of CRC patients.

Relationships between clinical stage-related gene expression and clinicopathologic factors

The relationships among GSR, CRLF1, AGPAT5, and NPR3 expression and clinical characteristics (N stage, T stage, M stage, and MSI) in CRC patients were assessed from the TCGA data set. Our results found that GSR and AGPAT5 expression levels were significantly negatively correlated with N stage and CRLF1 and NPR3 expression levels were significantly positively correlated with N stage in CRC (Figure 5A). CRLF1 and NPR3 expression levels were positively correlated with T stage in CRC, and AGPAT5 and GSR expression levels were negatively correlated with T stage (Figure 5B). CRLF1 and NPR3 expression levels were significantly positively correlated with M stage, and GSR expression levels were significantly negatively correlated with M stage in CRC (Figure 5C). For MSI, the expression levels of AGPAT5 and GSR were increased in the microsatellite instability – high (MSI-H) CRC group compared with the microsatellite stable ( MSS) CRC group (Figure 5D). CRLF1 and NPR3 expression levels were negatively correlated with TP according to the Pearson correlation coefficient in CRC (Figure 5E). These results revealed that cancer stage-related genes were correlated with cancer progression, MSI status, and TP in CRC.

AGPAT5 inhibited cell proliferation and migration, and induced cell apoptosis in CRC cells

The roles of GSR, CRLF1, and NPR3 in CRC cells have been reported in previous studies. However, the role of AGPAT5 in CRC cells is still unclear. We investigated the expression and role of AGPAT5 in a HIEC and CRC cell lines (HCT116 and SW480). First, RT-qPCR assays showed that the expression of AGPAT5 was significantly decreased in
HCT116 and SW480 cells compared with HIECs (Figure 6A). The overexpression plasmid pcDNA3.1-AGPAT5 and the control vector pcDNA3.1 were transfected into HCT116 and SW480 cells, respectively. RT-qPCR assays revealed that the expression of AGPAT5 was significantly increased in the OE-AGPAT5 group compared with the control group (Figure 6B). Overexpression of AGPAT5 significantly inhibited the proliferation of HCT116 and SW480 cells, as shown by the CCK-8 assay (Figure 6C). Next, we analysed the effect of AGPAT5 on the apoptosis of HCT116 and SW480 cells using flow cytometry. We found that overexpression of AGPAT5 increased the cell apoptotic rates (Figure 6D). In addition, a wound healing assay revealed that cell migration was reduced in the OE-AGPAT5 group compared with the control group after 48 h (Figure 6E). Collectively, these results suggested that overexpression of AGPAT5 decreased cell proliferation and migration, and promoted cell apoptosis in HCT116 and SW480 cells.

**Discussion**

In this study, four clinical stage-related genes (GSR, CRLF1, AGPAT5, and NPR3) were identified in CRC. The expression trends of GSR and AGPAT5 increased with cancer stage progression, and the expression trends of CRLF1 and NPR3 decreased with cancer progression in CRC patients. Higher GSR and AGPAT5 expression levels were associated with better OS, and lower CRLF1 and NPR3 expression levels were associated with better OS and DFS in CRC patients. Furthermore, GSR, CRLF1, AGPAT5, and NPR3 expression levels were related to cancer progression, MSI status and TP in CRC. In addition, overexpression of AGPAT5 decreased cell proliferation and migration, and promoted cell apoptosis in HCT116 and SW480 cells. The CNA of genes plays an important role in the developmental progression of CRC.22,23 Our results showed that genomic alterations of GSR, CRLF1, AGPAT5, and NPR3 occurred in 11%, 2.4%, 13%, and 3% of patients, respectively. Next, we found that AGPAT5 expression levels were negatively correlated with clinical stage, and high expression levels of AGPAT5 and GSR were independent poor prognostic factors.

Previous studies revealed that a high degree of heterogeneity of DNA CNAs was associated with a poor prognosis.22 The expression of AGPAT5 was negatively correlated with the N stage in CRC.22 To further investigate the influence of MSS on
Figure 2. Clinical stage-related genes were validated in the GSE41258 and GSE42284 data sets. (A) Heatmap of 53 positively related clinical stage genes in GSE41258. (B) Twelve common clinical stage-related genes were identified between GSE41258 and TCGA CAODREAD. Rows represent samples, including stages I, II, III, and IV. (C) Heatmap of 62 genes positively related to clinical stage in CRC. (D) The 16 common clinical stage-related genes were identified between GSE42284 and TCGA COADREAD. Rows represent genes, and green columns represent samples in clinical stages I, II, III, and IV. TCGA COADREAD indicates The Cancer Genome Atlas colorectal adenocarcinoma; CRC, colorectal cancer.
Clinical stage-related genes, we carried out the analysis according to TCGA-provided MSS information, including MSI-H and microsatellite low instability (MSI-L). We found that the expression of AGPAT5 in the MSI-H groups was significantly higher than that in the MSI-L groups. MSI status was also associated with AGPAT5 expression. Finally, we suggested that the expression of AGPAT5 was decreased in the CRC cell line and that overexpression of AGPAT5 decreased cell proliferation and migration and promoted cell apoptosis in HCT116 and SW480 cells. In CRC, AGPAT5 is regarded as a tumour suppressor gene involved in the proliferation, migration, and apoptosis of CRC cells.

Glutathione reductase (GSR) was reported to serve as a predictor for metastasis in CRC. A recent study found that high expression of AMPKα1 was correlated with a poor prognosis of CRC patients, and knockdown of AMPKα1 inhibited cell growth by regulating GSR phosphorylation. In this study, bioinformatics analysis found that the expression level of GSR was negatively correlated with clinical stage in CRC patients, and low expression of GSR was associated with poor prognosis in CRC patients. These results were consistent with those previously reported in lung cancer and breast cancer. The expression level of GSR was negatively correlated with the N and M stages in CRC. We found that the expression of GSR in the MSI-H groups was significantly higher than that in the MSI-L groups.

Cytokine receptor-like factor 1 (CRLF1) was found to be a downregulated and downstream target of miR-3065-3p in CRC.
Figure 4. Prognostic value of GSR, CRLF1, AGPAT5, and NPR3 in CRC. (A) GSR, CRLF1, AGPAT5, and NPR3 expression levels are associated with prognosis in the GEPIA database for CRC. (B) GSR, CRLF1, AGPAT5, and NPR3 expression are associated with prognosis on the PrognoScan website from the GEO database for CRC. GSR indicates glutathione reductase; CRLF1, cytokine receptor-like factor 1; CRC, colorectal cancer; GEPIA, gene expression profiling interactive analysis.
Figure 5. Relationships between GSR, CRLF1, AGPAT5, and NPR3 expression, and cancer progression, MSI status, and tumour purity in CRC. (A) Cancer stage-related gene expression levels are associated with N stage in CRC. (B) The CRLF1 and NPR3 expression levels were associated with T stage in CRC. (C) CRLF1, GSR, and NPR3 expression levels were associated with M stage in CRC. (D) AGPAT5 and GSR expression levels were associated with MSI status in CRC. (E) Scatter diagram showing the statistical results of the correlation of the CRLF1 and NPR3 expression levels with tumour purity analysed using the Pearson correlation coefficient in CRC (* \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \)). GSR indicates glutathione reductase; CRLF1, cytokine receptor-like factor 1; MSI, microsatellite instability; CRC, colorectal cancer.
Figure 6. Effects of OE-AGPAT5 on the proliferation, migration and apoptosis of HCT116 and SW480 cells. (A) RT-qPCR assays were used to detect the expression levels of AGPAT5 in HIEC, HCT116, and SW480 cells. (B) Transfection efficiency was detected by RT-qPCR assay. (C) A CCK-8 assay was used to detect the proliferation of HCT116 and SW480 cells. (D) A flow cytometry assay was used to detect the apoptosis of HCT116 and SW480 cells. (E) A wound healing assay was used to detect the migration of HCT116 and SW480 cells. Data are represented as the mean ± SEM (*<0.05, **<0.01, ***<0.001). RT-qPCR indicates reverse transcription-quantitative polymerase chain reaction; HIEC, human normal epithelial cell line; CCK-8, cell counting kit-8.
CRC cell lines, and overexpression of CRLF1 significantly decreased cell invasion and migration and inhibited the stemness of CRC cells. Recent analysis of the TCGA database revealed that CRLF1 may be a potential novel target for papillary thyroid carcinoma treatment. Herein, we found that the expression level of CRLF1 was positively correlated with the clinical stage in CRC patients, and the high expression of CRLF1 was an independent poor prognostic factor associated with shorter DFS and OS of CRC patients. The expression level of CRLF1 was positively correlated with the N, T, and M stages in CRC. TP, which was associated with poor prognosis, heavy mutation burden, and microenvironment, is defined as the proportion of cancer cells in the tumour tissue. We found that CRLF1 was negatively correlated with TP.

Upregulated NPR3 was correlated with low survival and candidate prognostic markers for CRC in stages II and IV. The expression of NPR3 was increased in CRC cell lines, and knockdown of NPR3 significantly inhibited CRC cell proliferation and viability and promoted cell apoptosis. In this study, our analysis suggested that the expression level of NPR3 was positively correlated with clinical stage in CRC patients. The expression level of NPR3 was positively correlated with N, T, and M stages, and negatively correlated with TP in CRC. A previous study reported that NPR3 might be a potential biomarker in breast cancer outcome prediction. Our study still had several limitations that need to be recognized. We will continue to follow up closely to validate the clinical meaning of GSR, CRLF1, AGPAT5, and NPR3 in further studies, and the functional analysis of GSR, CRLF1, and NPR3 needs to be explored in further studies using experimental methods. This was a retrospective study, and the TCGA database lacked some important clinicopathological features.

Conclusions
In summary, this study identified the expression of clinical stage-related genes in CRC progression, and low expression of AGPAT5 correlated with the clinical stage and prognosis of CRC patients. AGPAT5 decreased cell proliferation and migration, and promoted cell apoptosis in CRC cells. These findings have enriched our understanding of clinical stage-related genes, providing a valuable reference for the diagnosis and treatment of CRC in the future.

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Author Contributions
Jia Zang and Juanjuan Sun conceived and designed this study. WenChao Xiu wrote the manuscript. Xiaoshuang Liu collected data and analysed the data. Yunsheng Chai and Yanyan Zhou were responsible for the final modified version. The authors thank the authors of GEO and TCGA for making their data public for analysis.

Ethics Approval and Consent to Participate
This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for Publication
Not applicable.

Availability of Data and Material
Transcriptome data and clinical information of CRC patients could be downloaded from the TCGA data portal (http://cancer.gov/).

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.
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