REST Promotes Autophagy in Gastric Cancer by Transcriptionally Activating FABP6 to Inhibit the Akt/mTOR Signaling Pathway

Jing Luo1,2, Hongmei Yu1,2, Zhen Yuan1,2, Wenqing Tang3,*, Chen Wang1,2,*

1Department of Oncology, Minhang Branch, Zhongshan Hospital, Fudan University, 201199 Shanghai, China
2Key Laboratory of Whole-Period Monitoring and Precise Intervention of Digestive Cancer (SMHC), Minhang Hospital&AHS, Fudan University, 201199 Shanghai, China
3Department of Gastroenterology and Hepatology, Shanghai Institute of Liver Disease, Zhongshan Hospital, Fudan University, 200032 Shanghai, China

*Correspondence: tang.wenqing@zs-hospital.sh.cn (Wenqing Tang); wang_c@fudan.edu.cn (Chen Wang)

Academic Editor: Sung Eun Kim
Submitted: 28 January 2024 Revised: 20 April 2024 Accepted: 25 April 2024 Published: 12 June 2024

Abstract
Background: Gastric cancer (GC) is a leading cause of cancer-associated death worldwide. Its molecular mechanisms, especially concerning autophagy and various signaling pathways, are not fully understood. Fatty Acid Binding Protein 6 (FABP6) and RE1 Silencing Transcription Factor (REST) emerge as potential key players in this context. This study sought to analyze the functional relationship of FABP6 and REST concerning autophagy and their implications on the Akt/mTOR signaling pathway within GC cells. Methods: A comprehensive bioinformatics approach was used to identify key prognostic markers in GC. The effects of FABP6 and REST on autophagy along with Akt/mTOR signaling pathways were analyzed by techniques including Western blotting (WB), flow cytometry, Transwell assay, dual luciferase reporter assay, and others. Results: FABP6 was identified as overexpressed in GC, linked with poor prognosis. FABP6 silencing reduces GC cell proliferation, induces S- and G2-phase arrest, and downregulates cyclins CDK2 and CDK4. It also inhibited GC cell invasion/migration and autophagy, effects that were counteracted by MG132. When combined with PI3K inhibitor LY294002c, FABP6 knockdown showed synergistic anti-proliferative effects, modulating the Akt/mTOR pathway. Besides, the transcription factor REST has been shown to directly regulate FABP6 expression, affecting autophagy and the Akt/mTOR signaling pathway in a FABP6-dependent manner. Conclusions: REST positively regulates autophagy and negatively affects the Akt/mTOR signaling pathway in GC cells in a FABP6-dependent manner, providing valuable insights into regulatory networks involving FABP6 and REST.

Keywords: autophagy; gastric cancer; REST; FABP6; Akt/mTOR signaling pathway

1. Introduction

Gastric cancer (GC), also known as stomach cancer, is a major malignancy of the digestive system with various forms, of which the main subtype is stomach adenocarcinoma (STAD) [1,2]. Although its overall incidence has been decreasing, this trend is largely ascribed to lifestyle alterations such as diminished consumption of salt and preserved foods, along with an increased intake of fruits and vegetables, and a decline in smoking rates [3,4]. Advances in surgical methods, coupled with the application of chemotherapy, conventional radiotherapy, and neoadjuvant therapies, have enabled a high five-year survival rate for patients diagnosed at the early stages of the disease [5]. Due to the lack of symptoms in the initial phases of GC, most individuals receive a diagnosis only when the disease has reached an advanced or intermediate stage, leading to less-than-ideal five-year survival outcomes [6]. Given these barriers, there is an urgent need to discover unique indicators to help improve treatment strategies for patients with GC.

FABP6 is a specialized cytoplasmic protein primarily expressed in the small intestine and gallbladder [7,8]. Part of the Fatty Acid Binding Protein (FABP) family, FABP6 diverges from other cell death mechanisms by its principal involvement in the regulation of bile acid metabolism and intestinal absorption [9]. Functioning as a carrier for bile acids, it facilitates their translocation across enterocytes [8,10]. Recent studies have begun to unveil its potential role in cancer pathogenesis. Lian W et al. [11] pinpointed FABP6 as a candidate for enhancing immunotherapy in colorectal cancer, owing to its impact on immune infiltration, upregulation of major histocompatibility complex (MHC) class I expression, and recruitment of CD8+ T cells. Another investigation reported elevated FABP6 levels in primary colorectal cancer and adenomas, but a significant reduction in lymph node metastases, implicating its dual role in early carcinogenesis and modulation of tumor attributes such as aggressiveness, proliferation, and apoptosis [12]. Hu et al. [13] additionally validated the prognostic importance of FABP6 in liver hepatocellular carcinoma (LHHC). Yet, the role of FABP6 in gastric cancer remains elusive, warranting comprehensive studies to elucidate its implications.

Through rigorous analysis encompassing differential gene expression, pathway enrichment, and prognostic eval-
tations, this study aims to clarify the molecular underpinnings of GC while pinpointing possible avenues for treatment. Utilizing a comprehensive approach, we analyzed publicly available datasets, including the gene expression data from GSE103236, and integrated The Cancer Genome Atlas- Genotype-Tissue Expression (TCGA-GTEx) datasets, and corroborated these with our experimental results. Our analyses reveal that 11 key genes have significant prognostic value, and we further developed a six-gene risk model. Among these, FABP6 stands out as a critical prognostic marker, implicated in a diverse range of cellular activities including cell cycle progression, migration, invasion, as well as autophagy in GC cells. We also discovered its regulatory interaction with RE1 Silencing Transcription Factor (REST). Our findings not only broaden the current understanding of GC pathology but also lay the foundation for future studies focused on therapeutic interventions and personalized treatment strategies for GC.

2. Materials and Methods

2.1 Collection of Datasets

Gene expression data for the study were obtained from three different databases. The dataset GSE103236 was downloaded from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) database, comprising 9 normal gastric tissue samples adjacent to tumors and 10 GC samples. Additionally, 375 STAD samples were acquired from the TCGA (https://portal.gdc.cancer.gov/) database, and 359 normal control samples were obtained from the GTEx (https://gtexportal.org/home/) database. All datasets were normalized and preprocessed.

2.2 Differential Expression Analysis and Visualization

The “limma” package (version 3.40.2) within R software (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria) was utilized for analyzing gene expression differences. The fold change (FC) threshold for upregulated differentially expressed genes (DEGs) was set at FC >2, while for downregulated DEGs, it was set at FC <0.5. Only genes that met these criteria along with a p-value below 0.05 were deemed significant DEGs. The results were visualized using a clustering heatmap generated by the “pheatmap” package (version 1.0.12) and a volcano plot created using the “ggplot2” package (version 3.3.3) in R.

2.3 Identification of Overlapping DEGs

To identify overlapping upregulated and downregulated DEGs between the GSE103236 and the combined TCGA-GTEx datasets, the Venn diagram was generated using the “VennDiagram” package in R (version 3.3.3). Specifically, upregulated DEGs from GSE103236 were compared with upregulated DEGs from the TCGA-GTEx dataset to identify common upregulated DEGs. Similarly, downregulated DEGs from GSE103236 were compared with downregulated DEGs from the TCGA-GTEx dataset to identify common downregulated DEGs.

2.4 WikiPathway Enrichment Analysis

Following the identification of overlapping DEGs, these genes were subjected to WikiPathway enrichment analysis to understand their functional roles and involvement in different biological pathways. The “Enrichr” database (https://maayanlab.cloud/Enrichr/), a commonly used tool for gene-set enrichment analysis, was used for the research. p-values under 0.05 were used to determine if a pathway was significantly enriched. This methodological approach aims to synergize the findings from both datasets and offer insight on the mechanisms underlying the development of GC.

2.5 Integrated Survival and Crossover Analyses Identify Key Prognostic Genes in TCGA-STAD

A comprehensive bulk survival analysis focusing on Progression-Free Survival (PFS) was performed on all the genes expressed in the 375 TCGA-STAD samples. Genes were then stratified into two groups based on their impact on PFS: a good prognosis group and a poor prognosis group. p < 0.05 was set to identify genes that significantly affect PFS. Following the survival analysis, intersection analysis was conducted to identify key overlapping prognostic genes among the two prognosis groups, overlapping upregulated DEGs, and overlapping downregulated DEGs. This study was carried out in R using the “VennDiagram” package (version 3.3.3).

2.6 Prognostic Model Analysis of Key Overlapping Genes

The prognostic potential of 11 overlapping genes discovered through past survival and intersection investigations was assessed using the Least Absolute Shrinkage and Selection Operator (LASSO) Cox regression analysis. The optimal tuning parameter (λ) was determined through ten-fold cross-validation, aiming for the λ value that minimized the model deviance. The associated signature coefficients at this optimal λ were then calculated to evaluate the relative importance of each prognostic gene. According to the differential expression of signature prognostic genes, the STAD samples from the TCGA dataset were stratified into two risk groups (high-risk number 10, and low-risk number 108). Survival duration and condition among individuals in differing risk categories were juxtaposed, and the disparate gene expression within these categories was corroborated to affirm the risk stratification. For both risk groups, Disease-free survival (DFS) probabilities were calculated using Kaplan-Meier (KM) curves. The log-rank test was used to determine the significance of the DFS differences between the groups, and p-values were calculated to assess statistical significance. To further gauge the predictive accuracy of our risk model, we employed Receiver Operating Characteristic (ROC) curves. The Area Under the Curve...
(AUC) was determined for survival forecasts at 1, 3, and 5 years, serving as an assessment metric for the models’ prognostic capabilities.

2.7 Nomogram Analysis of Signature Prognostic Genes

After the signature prognostic genes were determined from the prognostic risk model, we then proceeded to conduct both univariate and multivariate Cox regression analyses using the “forestplot” package (version 1.10). Subsequently, the p-value, hazard ratio (HR), and 95% confidence interval (CI) were calculated. Following the univariate and multivariate Cox regression evaluations, we identified three key prognostic genes based on the p-value magnitude. Subsequently, a nomogram was generated, and the concordance index (C-index) was computed to assess the impact of these genes on patient survival at the 1-, 3-, and 5-year marks.

2.8 Expression in the University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN) Database and Analysis of KM Survival Curve

The UALCAN database (http://ualcan.path.uab.edu), which compiles information from the TCGA database, was used to determine the expression levels of FABP6 and REST in STAD samples. To validate the biological significance of FABP6 in STAD patients, KM survival curves were employed to evaluate its impact on various clinical outcomes, such as Post-Progression Survival (PPS), Overall Survival (OS), and First Progression (FP). We established a cut-off p-value below 0.05 to confirm statistical relevance.

2.9 Culture Conditions for Cells

Human gastric mucosal epithelial cells (GES-1) and GC cell lines (MKN45, BGC-823, SGC7901, MGC-803) were acquired from the FuDan IBS Cell Center in Shanghai, China. In addition, the Shanghai Cell Collection (Shanghai, China) provided HEK293T cells for dual-luciferase experiments. The cells were cultured referring to the literature of Thakore PI et al. [14]. All cell lines were validated by STR profiling and tested negative for mycoplasma. Cells were all cultured in a humidified incubator at 37 °C and 5% CO2.

2.10 Cell Treatments and Transfections

SGC7901 and MGC-803 cells were subjected to gene silencing using two different FABP6-targeted small interfering RNAs (siRNAs) (si-FABP6 #1 and si-FABP6 #2), with a non-targeting control siRNA (si-NC) acted as the reference control. The cells were seeded in 12-well plates at a concentration of 1.5 × 105 cells per well and transfected with siRNA the following day. Each well’s medium was replaced with 800 µL of serum-free medium, followed by the addition of a 200 µL mixture consisting of Opti-MEM (Opti-MEM I Reduced Serum Medium), 2.5 µL Lipofectamine 2000, and either 5 µL of negative control (NC) or si-FABP6 and si-REST. After gentle shaking, the plate was placed in the incubator. Following an 8-hour transfection period, the medium was substituted with 1 mL of complete medium containing serum and antibiotics. Cells were then harvested at 48 hours for subsequent experiments. For pharmacological treatments, cells underwent treatment with the proteasome inhibitor MG132 (10 µM) for 24 h. Additionally, the PI3K inhibitor LY294002 was applied to the cells. Cells were transfected with a REST overexpression construct or REST-targeted siRNA (si-REST) for overexpression and silencing of REST [15]. A non-targeting overexpression construct (over-NC) and a non-targeting siRNA (si-NC) served as controls for these manipulations.

2.11 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis and Primers Employed

We employed the qRT-PCR methodology according to previously described protocols [16]. In brief, RNA was extracted from SGC7901 and MGC-803 cells and subjected to cDNA synthesis. qRT-PCR was executed using specific primers for FABP6 and REST, with GAPDH as the control for normalization. Real-time PCRs were run for 40 cycles. The purity of both PCR and RT–PCR products were determined by single peak melting curves. Relative gene expression was computed via the 2^-∆∆Ct approach. The detailed primer sequences are as follows: FABP6 forward 5'-ACTACTCGGGGGCCACCAT-3' and reverse 5'-GTCTCTTGGCTACCGCTATAGG-3'; REST forward 5'-ACAGGATCTCTAGGAGCTCAGACTGG-3' and reverse 5'-CCAGGGTTCAGTTCTCTACACCAC-3'; and GAPDH forward 5'-GTGGTCTCTCTGACTTCAAC-3' and reverse 5'-CCACCAACCTCTTTGGCTGTA-3'.

2.12 Western Blotting (WB) Analysis

Protein expression levels were determined by WB after the indicated treatments and transfections. Cells underwent lysis in Radioimmunoprecipitation assay (RIPA) buffer (Biosharp, Guangzhou, China), which was enhanced with protease and phosphatase inhibitor additives. Following this, the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Shanghai, China) was employed to measure protein levels. Uniform protein quantities were subjected to Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) separation and subsequently electroblotted onto polyvinylidene difluoro (PVDF) sheets (Beyotime, Shanghai, China). Prior to overnight incubation with primary antibodies at 4 °C, these membranes were conditioned in a 5% skim milk solution. The following antibodies were used: anti-FABP6 (Abcam, ab96122, 1:1000, Shanghai, China), cell cycle-related proteins: anti-CDK2 (Abcam, ab32147), anti-CDK4 (Abcam, ab108357), anti-Cyclin A1 (Abcam, ab270940) and Cyclin A2 (Abcam, ab32386), all at 1:500; Migration and invasion markers :anti-Integrin β1 (Abcam, ab52971), anti-FAK (Abcam, ab81298), anti-p-FAK, anti-paxillin (Abcam, ab32084), anti-p-paxillin, anti-E-cadherin (Ab-
cam, ab233611), anti-N-cadherin (Abcam, ab254512), all at 1:800; Autophagy-related markers :anti-p62 (Abcam, ab109012), anti-LC3B-I (Abcam, ab192890), anti-LC3B-II (Abcam, ab232940), all at 1:700; Akt/mTOR signaling proteins: anti-p-mTOR, anti-p-Akt, anti-mTOR (Abcam, ab134903), anti-Akt (Abcam, ab38449), all at 1:600; anti-REST (Abcam, ab224744, 1:1000), and anti-GAPDH (Abcam, ab8245, 1:2000) served as an internal control. After washing, the membranes underwent treatment with HRP-conjugated secondary antibodies (Abcam, ab6728, 1:2000). Subsequent to chemiluminescent detection, protein band intensities were analyzed using ImageJ software (1.52s version, National Institutes of Health, Bethesda, MD, USA).

2.13 Cell Counting Kit-8 (CCK-8) Assay

The CCK-8 kit from Dojindo Laboratories (Dojindo, Kumamoto, Japan) was employed to gauge cell proliferation, following the guidelines provided by the supplier. In a nutshell, cells were allocated at a concentration of 4 × 10^4 cells in each well of a 96-well plate and cultured at 37 °C with 5% CO_2 in a moisture-controlled environment. At predetermined intervals (Days one through four after cell placement), a 10 µL volume of CCK-8 reagent was introduced into every well, succeeded by incubation for 120 minutes. Optical density (OD) at 450 nm was recorded using a microplate reader for the purpose of determining cellular viability.

2.14 Cell Cycle Assay

To assess cell cycle phase, treated and untreated cells were harvested by trypsinization, double washed with chilled phosphate-buffered saline, and stabilized overnight in 70% ethanol at 4 °C. After stabilization, the cells underwent another round of rinsing with chilled Phosphate Buffered Saline (PBS) and were then reconstituted in a 500 µL PBS solution enhanced with 50 µg/mL of propidium iodide (PI) and 100 µg/mL of RNase A. The cell suspension was incubated in the dark at room temperature for 30 minutes to allow for DNA staining. Flow cytometry was executed with a BD FACScanto II instrument (Becton, Dickinson and Company (BD) Biosciences, Franklin Lakes, NJ, USA), capturing no fewer than 1 × 10^4 events for each sample. Data interpretation was conducted through FlowJo software (Tree Star Inc, Stanford, CA, USA). Cells were categorized into G1, S, and G2 phases based on their DNA content, and the proportion of cells within each phase was quantified.

2.15 Transwell Analysis

Cells were seeded into 24-well Transwell plates with 8-µm pores (Corning Inc, Corning, NY, USA). 1 × 10^5 cells in serum-free media were put in the upper chamber for migration tests, while medium supplemented with 10% Fetal Bovine Serum (FBS) was added to the bottom chamber. For invasion assays, Matrigel-coated upper chambers were used and 2 × 10^5 cells were seeded. Cells that had migrated or infiltrated through the membrane were fixed and stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) after a 24-hour incubation at 37 °C in a 5% CO_2 environment. Microscopy was used to count the stained cells in five randomly selected fields at 100× magnification.

2.16 Identification of REST Binding Sites in the FABP6 Promoter Using JASPAR CORE Database

To predict the potential binding sites of REST within the FABP6 promoter region, we employed the JASPAR database (http://jaspar.genereg.net/). The genomic sequence of the FABP6 promoter was retrieved and input into the JASPAR database for computational analysis. The tool scanned the sequence for REST-specific motifs using a threshold score that is generally accepted for high-confidence prediction. Candidate REST binding sites were subsequently identified based on the output scores, and these sites were further analyzed for their possible roles in regulating FABP6 expression.

2.17 Luciferase-Based Transcriptional Activity Test

For this assay, HEK 293T cells underwent coinfection with either REST expression or control vectors, in conjunction with a plasmid featuring the FABP6 promoter and its potential REST binding region. Similarly, transfection of the expression constructs of transfer vector, packaging plasmids (pRSV-REV and pMDLg), and envelope plasmid (pCMV-VSVG) into HEK293T cells using Lipofectamine 2000 (Invitrogen, Shanghai, China) for subsequent assay. Two days after transfection, the cellular lysate was analyzed using a standard dual-luciferase protocol. Activity from firefly luciferase was adjusted relative to Renilla luciferase levels to account for variations in transfection efficiency.

2.18 Statistical Analysis of Experimental Data

We employed SPSS Version 26.0 (IBM SPSS statistics, Chicago, IL, USA) for statistical evaluation. Metrics are presented as mean ± standard deviation (SD), derived from no fewer than three separate trials. For comparing two sets of data, Student’s t-test was utilized, whereas one-way ANOVA with a Tukey’s post-hoc examination was used for multiple groups. The log-rank test was applied to assess survival differences based on Kaplan-Meier curves. A p-value below 0.05 denoted statistical relevance. Graphical representations were created through GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1 Wikipathway Enrichment Analysis of 731 Overlapping Genes Screened from GSE103236 and TCGA-GTEX Databases

As illustrated in Fig. 1A, we present the clustering distribution of the top 20 prognostic genes within
Fig. 1. Comprehensive analysis of differentially expressed genes (DEGs) and prognostic genes in Gastric cancer (GC). (A) Heat map, the cluster distribution of the top 20 genes with the most significant $p$ value in the normal control group (blue column) and tumor group (red column) in the GSE103236 dataset. (B) The volcano plot represents the DEGs in the The Cancer Genome Atlas-Genotype-Tissue Expression (TCGA-GTEx) database, where 2912 upregulated DEGs are marked with orange dots and 1601 downregulated DEGs are marked with purple dots. (C) Venn diagram, overlap between up- and down-regulated DEGs identified in the GSE103236 dataset and the TCGA-GTEx database. (D) Wiki pathway enrichment analysis of the 731 overlapping DEGs. The size of each bubble indicates the number of enriched genes, while the bubble color represents $-\log_{10} p$-value. (E) Forest plot of batch survival analysis in the TCGA-stomach adenocarcinoma (STAD) dataset, showing the top 20 genes with the most significant $p$-values associated with survival. (F) Venn diagram of overlapping genes found in the good prognosis group, the bad prognosis group, overlapping up-regulated DEGs, and overlapping down-regulated DEGs.

By leveraging the TCGA-GTEx database, we identified 2912 upregulated DEGs and 1601 downregulated DEGs (Fig. 1B). Further cross-referencing with the GSE103236-DEGs and TCGA-GTEx-DEGs led to the identification of 731 overlapping DEGs, which included 490 upregulated and 241 downregulated overlapping DEGs (Fig. 1C). Enrichment analysis showed that these overlapping DEGs were significantly related to Wiki pathways such as the IL-18 signaling pathway, Gastric Cancer Network 1, Focal Adhesion-Pi3K-Akt-mTOR signaling pathway, and Matrix Metalloproteinases (Fig. 1D).

### 3.2 11 Key Prognostic Genes Associated with STAD

Subsequently, by the results of the genome-wide prognostic analyses, we divided the prognostic genes of STAD into two groups: one group had 1388 genes associated with poor prognosis and the other group had 180 genes associated with better prognosis. Fig. 1E shows the top 20 genes with significant prognosis. From these categories (good prognosis group, poor prognosis group, upregulated overlapping DEGs, and downregulated overlapping DEGs), we further identified 11 key overlapping genes (Fig. 1F).

### 3.3 The Prognostic Power of a Six-Gene Risk Model in STAD Patients

We performed LASSO Cox regression analysis on 11 overlapping genes and determined coefficients for 6 eigen-genes according to lambda. $\min = 0.0272$ (Fig. 2A,B). On this basis, we established the following risk score formula: Risk score = $(0.0567) \times FABP6 + (0.0474) \times VCAN + (0.3586) \times CST6 + (0.0867) \times DKK1 + (0.0754) \times SERPINE1 + (0.2273) \times ONECUT2$. As shown in Fig. 2C, individuals in the high-risk cohort exhibited reduced survival durations, along with distinct expression profiles for these 6 genes across the two groups. In addition, the likelihood of DFS was markedly reduced for those in the high-risk cat-
Fig. 2. Evaluation of a six-gene prognostic model in STAD patients. (A) Least Absolute Shrinkage and Selection Operator (LASSO) Cox regression analysis of 11 overlapping genes, lines of different colors represent different genes. (B) Plot of Log(λ) versus partial likelihood deviation for determining a lambda value of 0.0272. (C) Risk model, the upper panel is the scatter trend of the low-risk group and the high-risk group, the middle panel is the survival time and status of patients in the two risk samples, and the lower heat map is the expression of characteristic prognostic genes in the two risk samples. (D) KM curves illustrating the differential survival outcomes between the cohorts at high and low risk. The y-axis represents survival probability, while the x-axis represents time. (E) Receiver Operating Characteristic (ROC) curve analysis of the risk model, the Area Under the Curve (AUC) values of the model at 1-year, 3-year, and 5-year intervals were 0.786, 0.715, and 0.748, respectively.

3.4 Identifying FABP6 as a Key Prognostic Marker

Following the LASSO Cox regression analysis, we subjected the six feature prognostic genes (FABP6, VCAN, CST6, DKK1, SERPINE1, ONECUT2) along with clinical variables (Age, Gender, pT-stage, pTNM-stage, Grade) to univariate and multivariate Cox regression analyses. Three genes emerged as significant prognostic factors, namely CST6, DKK1, and FABP6, each of which displayed \( p < 0.05 \), showing statistical significance (Fig. 3A,B). Utilizing these three genes, a nomogram (C index = 0.647) was developed to assess the survival prognosis of patients (Fig. 3C). Whereas the calibration curve of the nomogram showed that the model was closest to the calibration curve at 1 year, followed by 3 and 5 years (Fig. 3D). Among these three genes, the link between CST6 and DKK1 with GC is documented,
while the specific association of FABP6 with GC remains unclear. Therefore, in our follow-up study, we identified FABP6 as a hub gene for further study and further elucidated its role in GC.

3.5 FABP6 Contributes to Poor Prognosis in GC Patients

Analysis from the UALCAN database demonstrated that FABP6 was significantly overexpressed in TCGA-STAD samples (Fig. 4A). Further validation by in vitro experiments, including qRT-PCR and WB analyses, confirmed elevated expression of FABP6 in GC cell lines, particularly in SGC7901 and MGC-803, when compared to the GSE-1 control cell line (Fig. 4B,C). These findings point that the oncogenic role of FABP6 in GC. Prognostic evaluation indicated that individuals exhibiting reduced FABP6 levels experienced notably improved outcomes in terms of OS, PPS, and FP (Fig. 4D–F). Conversely, high expression of FABP6 correlated with poorer outcomes. Overall, this research underlined the potential significance of FABP6 as a prognostic indicator and emphasize its relevance in understanding disease progression and patient outcomes.

3.6 Silencing of FABP6 Inhibits Cell Proliferation and Impedes Cell Cycle Progression in GC Cells

We assessed the knockdown efficiency of si-FABP6#1 and si-FABP6#2 through qRT-PCR and WB analyses and found that si-FABP6#2 significantly reduced the expression levels of FABP6 (Fig. 5A–C). CCK-8 assays were performed on SGC7901 and MGC-803 cells transfected with si-FABP6#2 to evaluate the impact of FABP6 silencing on cell proliferation. Over time, a marked decrease in cell viability was observed (Fig. 5D,E). Further examination by
Fig. 4. Association of FABP6 expression levels with prognostic indicators in GC. (A) Analysis of FABP6 expression levels in TCGA-STAD samples compared to normal gastric tissue samples, as assessed by the University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN) database. (B,C) The bar graph in the upper figure is the relative mRNA expression level of FABP6 in control cells and GC cell lines analyzed by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). The lower strip diagram shows the protein expression level of FABP6 detected by Western blotting (WB) in control cells and GC cell lines. The bar graph showed the results of the gray-scale detection of proteins. *p < 0.05, **p < 0.01. (D–F) KM survival curves illustrate the prognostic impact of FABP6 expression levels on overall survival (OS) (D), post progression survival (PPS) (E), and first progression (FP) (F) in GC patients. The months are measured on the x-axis and the survival probability is shown on the y-axis. Log-rank test results indicate statistical significance.

Flow cytometry showed that administration of si-FABP6#2 resulted in a decrease in cells in the G1 phase, along with an accumulation of cells in the S and G2 phases (Fig. 5F–I). Moreover, WB assessment revealed notable decreases in proteins related to cell cycle regulation, such as CDK2 and CDK4, upon FABP6 knockdown (Fig. 5J). The concentrations of Cyclin A1 and A2 remained largely unaltered. These data implied that FABP6 is important for GC cell proliferation and cell cycle progression.

3.7 FABP6 Knockdown Suppresses Migration and Invasion of GC Cells

Transwell experiments were used to determine the involvement of FABP6 in the migration and invasion of GC cells (Fig. 6A–D). The results showed that silencing FABP6 led to a notable decrease in both migrating and invading cells within GC cell lines when contrasted with the control group. For a more comprehensive understanding of the molecular pathways involved, we scrutinized alterations in the levels of proteins linked to cell migration and invasion after inhibiting FABP6 in both cell lines (Fig. 6E–G). Specifically, we observed downregulation in the expression of p-FAK, integrin β1, and p-paxillin, indicating impaired focal adhesion signaling. Moreover, an increase in the protein expression of E-cadherin and a decrease in the expression level of N-cadherin were found in FABP6-silenced cell lines. Therefore, we believe that FABP6 plays a key role in regulating GC cell migration and invasion, potentially through modulating focal adhesion and cadherin signaling pathways.

3.8 FABP6 Knockdown Promotes Autophagy and Inhibits Proliferation in GC Cells

To explore how inhibiting FABP6 affects autophagy in GC cells, we evaluated the abundance of autophagy-linked proteins through WB (Fig. 7A). The findings showed the diminished abundance of p62 as well as LC3B I/II, indicating reduced autophagy following FABP6 knockdown. To further explore the degradation system involved in this regulation, we utilized MG132, a proteasome inhibitor [17,18]. Interestingly, the addition of MG132 effectively counteracted the decline in protein expression induced by FABP6 knockdown (Fig. 7B–D). Subsequently, we investigated the impacts of MG132 treatment and FABP6 knockdown on...
Fig. 5. Functional assessment of cell viability, cell cycle distribution, and cell cycle-associated proteins by FABP6 silencing in GC cells. (A–C) The efficiency of FABP6 knockdown was validated by qRT-PCR and WB analyses in SGC7901 and MGC-803 cells. The y-axis represents relative gene or protein expression levels, while the x-axis represents the different siRNA conditions. The bar graph showed the results of the gray-scale detection of proteins. (D,E) Cell Counting Kit-8 (CCK-8) assays show the inhibitory effects of si-FABP6#2 on cell viability over time in SGC7901 and MGC-803 cells. The y-axis represents the cell viability percentage, and the x-axis represents the time points in days. (F–I) Flow cytometry of cell cycle distribution in SGC7901 and MGC-803 cells transfected with si-FABP6#2. The G1, S, and G2 stages of the cell cycle are represented by the peaks in the left image, where the x-axis measures DNA content and the y-axis measures cell numbers. The bar graph on the right is the percentage of cells in different G1, S, and G2, where the y-axis indicates the cell cycle rate (%) in each phase and the x-axis indicates the treatment conditions of the cell lines in different phases. (J) WB analysis of cell cycle-associated proteins (CDK2, CDK4, and Cyclin A1+A2) after FABP6 silencing. NC, negative control; *p < 0.05, **p < 0.01.

GC cell viability using CCK-8 assays (Fig. 7E,F). Our results showed that both transfection with si-FABP6#2 and MG132 treatment led to a decline in cell viability, and their combined effects further intensified the inhibition of cellular proliferation.

3.9 FABP6 Knockdown and LY294002c Treatment Synergistically Inhibit GC Cell Proliferation Viability

Considering the pivotal function of the Akt/mTOR signaling pathway in controlling cellular growth, survival, as well as metabolism [19], we aimed to investigate how this pathway is influenced by FABP6 knockdown in GC
Fig. 6. Impact of FABP6 silencing on GC cell migration, invasion, and related protein levels. (A–D) Transwell assays demonstrating the impact of FABP6 knockdown on cell migration and invasion in GC cell lines. Scale bar = 50 μm. (E–G) WB analysis evaluating the expression levels of key proteins involved in cell migration and invasion following FABP6 knockdown. The x-axis specifies the treatment conditions, and the y-axis indicates relative protein expression. The bar graph showed the results of the gray-scale detection of proteins. *p < 0.05.

3.10 REST Directly Regulates FABP6 Expression by Binding to Promoter Sites

We first examined the TCGA-STAD dataset to investigate the regulatory connection between REST and FABP6 in GC. REST expression was shown to be considerably increased in GC tissues, implying an oncogenic function (Fig. 9A). Subsequent WB analyses post-REST knockdown indicated a decrease in FABP6 expression levels in GC cell lines, corroborating a positive regulation of FABP6 by REST (Fig. 9B). To identify the possible binding sites of REST in the promoter region of FABP6, we employed the JASPAR database for in silico prediction. Three putative binding sites were identified: bind site1 (TAGCTGGGC-CTGGTGTGTC), bind site2 (AGGGTGTCCAGGGC-CTGG), and bind site3 (GATCTCACCATGTGTGCCC).
Fig. 7. Effects of FABP6 knockdown and MG132 treatment on autophagy-related protein expression and cell viability in GC cells. (A) WB detection of autophagy-related protein expression changes in SGC7901 and MGC-803 cells after FABP6 knockdown. (B–D) WB detection of the effect of knocking down FABP6+MG132 on the expression levels of autophagy-related proteins in GC cells. The bar graph showed the results of the gray-scale detection of proteins. (E,F) CCK-8 assay assesses cell viability after FABP6 knockdown and MG132 treatment in SGC7901 and MGC-803 cells. *p < 0.05 vs. si-NC; #p < 0.05 vs. si-NC+MG132.

Fig. 8. Regulation of cell viability by FABP6 knockdown and PI3K inhibition in GC cells. (A) WB detection of expression levels of Akt/mTOR signaling pathway-related proteins after FABP6 knockdown. (B,C) The CCK-8 assay examined the individual and combined effects of FABP6 knockdown and the PI3K inhibitor (LY294002c) on cell viability in GC cells. *p < 0.05.

(Fig. 9C). To experimentally validate these inferences, we conducted dual-luciferase reporter assays (Fig. 9D,E). Our data showed that overexpression of REST significantly enhanced luciferase activity at the FABP6 binding sites, while mutations at these sites abolished this interaction. Collectively, our findings provided compelling evidence that REST regulated FABP6 levels through direct interactions with gene promoter regions.

3.11 REST Promotes Autophagy by Transcriptionally Activating FABP6 to Inhibit Akt/mTOR Signaling Pathway

We evaluated the efficiency of REST overexpression in GC cells using quantitative RT-PCR and WB. Our results demonstrated a significant upregulation of REST in the GC cells (Fig. 10A,B). Following REST overexpression, WB data indicated elevated levels of autophagy-
Fig. 9. REST directly regulates FABP6 expression. (A) The UALCAN database detects the expression of REST in GC, based on the TCGA-STAD dataset. (B) WB detection of FABP6 protein level and REST knockdown efficiency in GC cell lines after REST knockdown. (C) JASPAR database predictions identified REST binding sites within the FABP6 promoter. (D) Schematic of the dual-luciferase reporter construct with the wild-type REST binding site on top and the mutated REST binding site on the bottom. (E) Dual luciferase activity assay overexpression of REST has luciferase activity on the FABP6 binding site. WT, wild type; MT, mutant. *p < 0.05.

associated proteins, including p62, LC3B-I, and LC3B-II (Fig. 10C). Conversely, we observed diminished levels of crucial molecules in the Akt/mTOR pathway, specifically p-Akt and p-mTOR (Fig. 10D). Interestingly, in the context of REST overexpression along with FABP6 knockdown in GC cells, a decline was observed in the expression levels of autophagy-associated proteins. Additionally, we noted an increase in the levels of key proteins associated with the Akt/mTOR pathway (Fig. 10E–G). These observations suggested that REST positively regulated autophagy in a FABP6-dependent manner, while concurrently inhibiting the Akt/mTOR signaling pathway.

4. Discussion

GC remains a globally prevalent malignancy, presenting significant challenges in diagnosis, targeted gene therapy, and long-term survival outcomes [20]. Current diagnostic protocols for GC are primarily reliant on a combination of clinical symptoms, endoscopic examinations, histopathological analyses, and imaging techniques [21, 22]. While these modalities are indispensable, their limitations include late-stage detection and reduced sensitivity, highlighting a pressing need for more robust diagnostic tools. Targeted gene therapies, aimed at the specific inhibition of key oncogenes or the activation of tumor suppressor genes, offer promising avenues for GC treatment [23, 24]. Yet, the identification of effective target genes continues to be a hurdle in enhancing therapeutic outcomes. Furthermore, the five-year survival rate for GC varies substantially according to the stage at which the disease is diagnosed. In early-stage GC, where the tumor is confined to the mucosal or submucosal layers, the survival rate can exceed 90% [25, 26]. However, this drops precipitously to below 30% for advanced cases with distant metastases [27]. This highlights the urgent need for new prognostic indicators and alternative treatment options to improve patient survival. In this context, investigating the role of FABP6 in the diagnosis, treatment of GC holds significant promise.

Our study delineated a complex network of interactions among various prognostic genes and pathways in STAD, with FABP6 emerging as a notably significant player. The six-gene risk model, which incorporates FABP6, exhibits potent predictive capabilities with AUC values exceeding 0.7 for survival rates at 1, 3, and 5 years, indicating its robustness as a prognostic tool. It’s also noteworthy that CST6 and DKK1 have been previously implicated in GC. For instance, CST6 is upregulated in the necroptosis-related genes prognostic index-high subgroup of STAD patients and is related to poor prognosis [28]. Similarly, DKK1 has been shown to correlate with poorer sur-
Fig. 10. REST overexpression and FABP6 knockdown differentially regulate autophagy and Akt/mTOR signaling pathway in GC cells. (A and B) qRT-PCR and WB analyses showed significant REST overexpression in GC cells. *p < 0.05. (C) WB detection of REST overexpression and expression of autophagy-related proteins in GC cells. (D) WB detected the regulation of REST overexpression on the protein levels of Akt/mTOR signaling pathway in GC cells. (E–G) WB detected autophagy-related proteins and Akt/mTOR pathway signaling-related proteins in GC cells following over-REST+si-FABP6#2 treatment. The bar graph showed the results of the gray-scale detection of proteins. *p < 0.05, **p < 0.01 vs. si-NC; #p < 0.05, ###p < 0.01 vs. si-FABP6#2 and si-NC+MG132.

Vival rates in GC patients, through mechanisms that involve the creation of an immunosuppressive tumor microenvironment [29]. Literature also suggests that DKK1 is targeted by anesthesia-induced miR-493-3p, which inhibits GC cell growth by repressing Wnt/β-catenin signaling and is implicated in GC cell invasion, proliferation, and drug resis-
stance [30]. Given these precedents, \textit{FABP6} was selected as the focal prognostic marker for our study. Its overexpression in TCGA-STAD samples and correlation with poor patient outcomes underscore its potential utility as a biomarker. Given that current targeted gene therapies often hit roadblocks due to a lack of effective target genes, these insights could be groundbreaking. Therefore, further investigations into the specific roles and regulatory mechanisms of \textit{FABP6} could significantly advance the prognosis and treatment strategies for GC.

In addition to validating \textit{FABP6} as a pivotal factor in the prognostic landscape of GC, our study elucidated its multiple functional roles. We confirmed that silencing \textit{FABP6} significantly impedes cell proliferation as well as alters cell cycle progression in GC cells. The downregulation of cell cycle-associated proteins like CDK2, and CDK4 suggests a specific mechanism through which \textit{FABP6} affects cellular processes. Additionally, \textit{FABP6} knockdown disrupts migration and invasion, potentially through modulating focal adhesion and cadherin signaling pathways. Autophagy serves as a double-edged sword in cancer progression, allowing cells to survive or die depending on the circumstances [31,32]. For instance, autophagy may enhance drug resistance in some cancers while inducing cell death in others [33]. In our study, a decline in autophagic activity was observed following \textit{FABP6} knockdown, as indicated by diminished levels of markers such as p62 and LC3B forms I and II. When investigating the degradation systems responsible for this autophagy modulation, we found that the proteasome inhibitor MG132 counteracts the decline in autophagy-related protein expression caused by \textit{FABP6} knockdown. Interestingly, the addition of MG132 not only restored the protein levels but also exacerbated the anti-proliferative effects of \textit{FABP6} knockdown. This suggests that proteasomal degradation pathways may be crucial cofactors in the \textit{FABP6}-mediated regulation of GC cell dynamics.

The Akt/mTOR signaling system plays an important role in cancer cell proliferation, metabolism, and survival [34,35]. For instance, the pathway is known to promote proliferation and migration in GC cells and is regulated by elements like circNR3C1 levels [36]. Increased expression of PD-L1 and its binding partners, PD-L1 and PD-L2, coupled with activity in the Akt/mTOR pathway, is associated with GC progression and distant metastasis [37]. Conversely, tumor suppressors like \textit{TOb1} and \textit{E2F2} overexpression have been shown to inhibit this pathway, triggering mechanisms like autophagy to counteract GC progression [38]. In our study, \textit{FABP6} knockdown surprisingly led to the upregulation of p-Akt and p-mTOR, key components of the Akt/mTOR signaling pathway. This suggests that the anti-proliferative effects of \textit{FABP6} silencing might not operate solely through this pathway or could potentially activate a compensatory mechanism. To further understand the role of this pathway, we introduced the PI3K inhibitor LY294002c. Both \textit{FABP6} knockdown and LY294002c treatment individually reduced cell viability, but when combined, their effects were synergistic, showing an even greater decline in cellular viability. This suggests a complex interplay between \textit{FABP6} and the Akt/mTOR pathway, where the PI3K inhibitor can potentiate the anti-proliferative effects of \textit{FABP6} knockdown.

In the context of the prior discussion highlighting the intricate role of \textit{FABP6} and the Akt/mTOR signaling pathway in GC, our findings on \textit{REST} added another layer of complexity. \textit{REST} is a neuron-restrictive silencer factor known for activating autophagy [39,40]. Its absence has been linked to autophagy failure, loss of protein homeostasis, increased oxidative stress, and elevated cell mortality [41]. While the therapeutic potential of \textit{REST} has been explored in diseases such as castration-resistant prostate cancer [42], its role in GC has not been previously reported. Our data indicate that \textit{REST} is significantly upregulated in GC tissues and directly regulates \textit{FABP6} by binding to its promoter region. This suggests a hierarchical interaction wherein \textit{REST} acts upstream of \textit{FABP6}. Interestingly, \textit{REST} overexpression led to elevated levels of autophagy-associated proteins and a simultaneous downregulation of Akt/mTOR signaling components. However, when \textit{REST} was overexpressed with \textit{FABP6} knockdown, the effect was reversed, suggesting that \textit{REST} regulation of autophagy and the Akt/mTOR pathway is \textit{FABP6}-dependent. These findings place \textit{REST} as a pivotal player in a regulatory network that includes \textit{FABP6} and the Akt/mTOR pathway. The ability of \textit{REST} to promote autophagy and inhibit Akt/mTOR signaling in an \textit{FABP6}-dependent manner suggested a multi-tiered regulatory mechanism. This opened new avenues for targeted therapy, especially given the established role of \textit{REST} in other cancers, thereby highlighting its potential as a multifunctional therapeutic target in GC.

5. Conclusions

In conclusion, our comprehensive study elucidated the complex regulatory network involving \textit{FABP6}, the Akt/mTOR signaling pathway, and \textit{REST} in the context of GC. We demonstrated that \textit{FABP6} knockdown inhibits GC cell proliferation and viability, implicating it as a potential oncogene. Intriguingly, our results showed that its downregulation impacts the Akt/mTOR signaling pathway, a crucial regulator of cell growth and survival. We also found that combining \textit{FABP6} knockdown with \textit{PI3K} inhibitor LY294002c resulted in synergistic inhibition of GC cell viability. Importantly, our research was the first to introduce the role of \textit{REST} in GC. \textit{REST} not only directly upregulated \textit{FABP6} but also acted as a regulatory switch for both autophagy and Akt/mTOR signaling in a \textit{FABP6}-dependent manner. These findings offered novel insights into GC pathology and opened new avenues for targeted therapeutic strategies.
6. Limitation

Despite the reliability of our findings, there are inherent limitations to this study. First, our study relied heavily on in vitro experiments involving GC cells, but it may not fully reflect the complexity of human gastric organs in vivo. Second, although our findings suggest a regulatory relationship between FABP6, the Akt/mTOR signaling pathway, and REST, determining causality requires more extensive studies. There are some limitations to this study in vivo and in the clinical applications. Finally, potential feedback loops and other intermediate factors regulating ion channel expression remain to be explored, necessitating a more comprehensive understanding of the regulatory network.

Availability of Data and Materials

The data supporting the findings of this study are available from the corresponding author upon reasonable request. The datasets analyzed during the current study are available in the following public repositories: The Gene Expression Omnibus (GEO) database houses the gene expression dataset GSE103236, which is available at https://www.ncbi.nlm.nih.gov/geo/. The Cancer Genome Atlas (TCGA) provided the STAD samples, accessible at https://portal.gdc.cancer.gov/ . Normal control samples were retrieved from the Genotype-Tissue Expression (GTEx) database, found at https://gtexportal.org/home/ . The Enrichr database facilitated Wikipathway enrichment analysis and is available at https://maayanlab.cloud/Enrichr/ . Expression levels and survival analyses involving STAD samples were performed using the UALCAN database, available at http://ualcan.path.uab.edu . The JASPAR database was employed for predicting potential REST binding sites within the FABP6 promoter region, which can be accessed at http://jaspar.genereg.net/ .

Author Contributions

JL and HY designed the research study. ZY performed the research. WT and CW analyzed and interpreted the data. CW wrote the manuscript and revised it critically for important intellectual content. All authors contributed to editorial changes in the manuscript. All authors approved the final submitted version. All authors agree to be accountable for all aspects of the work to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

References
