E2F2 Inhibition Induces Autophagy to Impair Metastasis via the PI3K/Akt/mTOR Pathway in Gastric Cancer

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Abstract

Background

E2F2 is a member of the E2F transcription factor family and has important but not fully understood biological functions in cancers. The pro- and antitumor functions of E2F2 in some cancer types are controversial. However, the biological role of E2F2 in gastric cancer (GC) remains unclear.

Methods

E2F2 expression from multiple gene expression databases was analyzed. Kaplan-Meier plots and Cox regression were used to analyze the prognostic value of E2F2 expression. The correlation between E2F2 and tumor immune infiltration was investigated using the Tumor Immune Estimation Resource (TIMER) database. The functions and pathways of E2F2 and its 50 most highly correlated genes in terms of expression pattern were analyzed using Database Annotation, Visualization, and Integrated Discovery (DAVID) software. We used immunohistochemistry, real-time quantitative polymerase chain reaction, and western blotting to detect the expression level of E2F2 in GC. We further investigated the effects of E2F2 on phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling, autophagy, and the migration and invasion of GC cells by the wound healing assay, Transwell assay, western blotting, and transmission electron microscopy.

Results

E2F2 was highly expressed in both GC tissues and cells compared with normal gastric tissues/cells in public datasets and our validation experiments. High E2F2 expression was associated with poor overall survival (OS). In addition, the expression of E2F2 in GC had a strong correlation with a variety of immune markers. E2F2 overexpression promoted the migration and invasion of GC cells in vitro through the inhibition of PI3K/Akt/mTOR-mediated autophagy. In contrast, inhibition of E2F2 inhibited the migration and invasion of GC cells in vitro by activating PI3K/Akt/mTOR-mediated autophagy.

Conclusion

This study provides multilevel evidence for the significance of E2F2 in the pathogenesis of GC and its potential as a biomarker for GC. E2F2 was highly expressed in GC, and high E2F2 expression was associated with the characteristics of invasive tumors and poor prognosis. E2F2 had potential modulatory effects on tumor immunity. We found a novel function of E2F2 in the regulation of PI3K/Akt/mTOR-mediated autophagy and the downstream process of cell migration and invasion. Our results may provide novel targets and strategies for the diagnosis and treatment of GC.

Background

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer-related death (1). Due to the high recurrence rate and metastasis rate, the five-year survival rate of advanced GC

is very low (2). The existing targeted drugs show unsatisfactory efficacy, which is due to the combination of different clinical and biological factors, as well as the development of drug resistance by the GC (3). The molecular mechanism of tumor formation and progression is still unclear, which further complicates the effective treatment of GC (4). In addition, the lack of specific markers targeting tumor types or disease stages is still an important gap in the understanding and treatment of GC.

E2Fs are a transcription factor protein family that are major regulators of cell growth and proliferation. The main functions of the E2F proteins are to regulate the cell cycle, cell differentiation, DNA damage response, and cell death (5). The biological role of E2F2 in GC remains largely unknown, although some members of the E2F family, such as E2F1, have been extensively studied (6-9). E2F2 plays contradictory roles in the occurrence and development of tumors. On the one hand, E2F2 can inhibit tumorigenesis by inhibiting cell cycle regulators (10). On the other hand, it can act as an activator to increase the expression of target genes, leading to cancers (11). In GC, whether E2F2 acts as an oncogene or as a tumor suppressor gene is worthy of further study. This study aimed to fill the gap in the understanding of the role of E2F2 in the occurrence and/or progression of GC and lay the foundation for the development of novel treatment strategies for GC.

More than 90% of cancer deaths are caused by tumor metastasis, and there is growing evidence that autophagy is closely associated with tumor metastasis (12, 13). Autophagy is a highly conserved catabolic process that maintains intracellular environmental stability by directing damaged proteins or organelles to the lysosome compartment for degradation (14, 15). This process is important both in normal human physiology and in diseases, including cancers (16). In GC, inhibition of autophagy is associated with the promotion of cell proliferation, migration, and invasion, which may involve the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway (17). Although autophagy and the PI3K/Akt/mTOR pathway seem to play key roles in the metastasis of GC, it is unclear whether E2F2 may play a mediating role in these processes.

Here, we studied E2F2 expression and mutations in GC patients from The Cancer Genome Atlas (TCGA) and various public databases. Using multidimensional analysis, we evaluated the E2F2-related genomic alterations and functional networks in GC and explored their role in tumor immunity. Finally, we studied GC cell lines to validate the high expression of E2F2 in GC and examined the function of E2F2 in GC cell invasion, migration, and autophagy. Our data show that E2F2 mediated autophagy through the PI3K/Akt/mTOR pathway, revealing a novel role for E2F2 in GC cell metastasis.

Methods

1.ONCOMINE database

The ONCOMINE Database (www.oncomine.org) is an integrated online cancer microarray database that contains data from DNA sequencing and RNA sequencing (DNA-seq and RNA-seq, respectively) analysis, which are used to classify differential expressions between common cancer types and cancerous types. Corresponding normal tissue and clinical and pathological data. (18). In our study, the transcriptional

expression data for E2F2 in different cancer tissue samples and their corresponding adjacent normal samples were obtained from the ONCOMINE database. Differences in transcriptional expression were compared by Student's t test. The p-value cutoff and fold change threshold were as follows: p-value, 0.01; fold change, 1.5; gene grade, 10%; and data type, mRNA.

2.UALCAN database

UALCAN (<http://ualcan.path.uab.edu>) is an interactive network resource developed based on level 3 RNA sequences and clinical data for 31 cancer types in the TCGA database (19). In this study, UALCAN was used to analyze the mRNA expression of E2F2 in primary gastric cancer tissue samples and the relationship between these members and clinicopathological parameters. The differences of transcriptional expression were compared by students't-test ($p < 0.01$).

3.GEPIA database

The (GEPIA) database for interactive analysis of gene expression profiles (<http://gepia.cancer-pku.cn/>) is an interactive Web, containing 9736 tumors and 8587 normal samples from the TCGA and GTEx projects (20). In this study, GEPIA was used to verify the expression of E2F2 in GC.

4.LinkedOmics Database

The LinkedOmics database (<http://www.linkedomics.org/login.php>) is a Web-based platform for analyzing 32 cubes related to TCGA cancer (21). The Pearson co-expression was statistically analyzed by using E2F2 correlation coefficient, and displayed in the form of volcano map, heat map or scatter diagram. The rank criterion was $FDR < 0.05$ and 1000 simulations were performed.

5.cBioPortal Database

cBioPortal includes 478 gastric cancer pathology reports data from TCGA (22). The genome map includes mutations, copy number changes from GISTIC (CNA), mRNA expression z score (RNA-seq V2 RSEM) and protein expression z score (RPPA). The E2F2 mutation, copy number variation (CNV) and gene co-occurrence in GC were analyzed by c-BioPortal tool.

6.TIMER database

TIMER (<https://cistrome.shinyapps.io/timer/>) is a comprehensive resource for systematic analysis of immune infiltration in various cancer types, including 10897 samples from 32 cancer types (23). TIMER used deconvolution method (24) to infer the abundance of tumor infiltrating immune cells (TIIC) from the gene expression profile. We analyzed the expression of E2F2 in STAD and the correlation between the expression of E2F2 and the content of immune infiltrating fluid.

7.Protein-protein interaction (PPI) network construction and gene enrichment analyses

In this study, the STRING database (25) was used to analyze E2F2 and 50 frequently changing genes closely related to family members. We used the annotated, visualization and comprehensive discovery database (DAVID) (<http://www.DAVID.org>) (26) to conduct an (KEGG) analysis of the motivational gene ontology (GO) and the Kyoto Encyclopedia of genes and genomes. GO enrichment analysis can predict gene function according to the composition of (CC) and molecular function (MF) of (BP), cells in biological process, while KEGG can be used to analyze the pathway of gene enrichment.

8.Tissue microarrays (TMAs)

We collected 60 fresh GC tissues and adjacent tissues for IHC staining and evaluation. The staining intensity was given a score from 0 to 3 based on the following criteria: 0, no staining; 1, weak staining; 2, moderate staining; or 3, strong staining. All individual patients understood the objectives of this study and provided written informed consent. The Medical Ethics Committee of Qingdao University and the Affiliated Hospital of Qingdao University approved the collection of clinical materials for research purposes.

9.Cells and culture conditions

AGS and HGC27 cell lines were purchased from the cell bank of the Chinese Academy of Sciences and were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Gibco, NY, USA). The cells were placed in an incubator at 37 °C with a CO₂ concentration of 5%.

10.Transfection

Cells were transfected with plasmids expressing E2F2 (GV141-E2F2, Genechem, Shanghai, China), plasmids expressing empty vector (GV141-Vector), small interfering RNAs (siRNAs) against E2F2 (siE2F2; GenePharma, Shanghai, China, Supplementary Table 1) and negative control (siNC) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

11.RNA extraction and qPCR

RNA was extracted from cells via Trizol (TaKaRa). 1 µg of this isolated RNA was then used to generate cDNA using 5 × primer script buffer, primer script enzymes, oligo dT primers and reverse transcriptase (TaKaRa). cDNA was generated through a reverse transcription reaction which was conducted at 37 °C for 15 min, 84 °C for 5 s and 4 °C for the appropriate amount of time. Real-time quantitative PCR (qPCR) was carried out using SYBR Green PCR master mix. Gene expression was normalized against β-actin. Each experiment was repeated three times and was performed independently in duplicate. mRNA expression was quantified by the cycle threshold (CT) method, and SPSS version 11.5 was used to calculate identify significant differences in the mRNA expression levels of various genes among different samples using the Mann-Whitney U test. The PCR primers used are listed in Supplementary Table 1.

12.Western blotting analysis

Western blotting analysis of protein expression was performed as described previously (27). Briefly, protein lysates (20 µg) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and target proteins were detected by western blotting with antibodies against E2F2 (1:1000) and GAPDH (1:50,000). Other antibodies used in this study are listed in Supplementary Table 1.

13. Electron microscopy

The cells were fixed at 4 °C with 2.5% glutaraldehyde (Solarbio, Beijing, China). H, washed with phosphate buffer (PBS) and fixed with 1% OsO₄ buffer at 4 °C. H. The cells were then washed and dehydrated in a series of graded ethanol solutions and embedded in Epon812 epoxy resin. Ultra-thin (90 nm) sections were collected on copper net, stained with 1% uranyl acetate and 0.2% lead citrate, and examined by JEOL-1200EX transmission electron microscope (Beijing, China).

14. Cell migration and invasion assays

Cells in logarithmic phase were starved in serum-free medium 1640 for 24 hours, then digested with 0.25% EDTA-trypsin. The cell suspension was then treated with serum-free 1640, and 200 µ L of cells were added to the upper chamber of the Transwell insert (Corning Costar), while the complete growth medium containing serum was added to the lower chamber at a rate of 600 µ L / L. After 24 hours, the cells were fixed with methanol for 20 minutes, and then stained with crystal violet for 20 minutes after drying. Use a wet swab to gently remove the cells retained in the upper chamber, and then place the chamber under an inverted microscope so that the remaining cells can be counted. The images were analyzed by ImageJ software.

15. Wound-healing assay

We inoculated the cells into a 6-well plate and then used a 200 µ L pipette suction head to form a wound on the monolayer surface of the fused cells. The cells were then washed with PBS and incubated in serum-free medium for 24 hours. Since then, we have captured images of the cells at different times within 24 hours. The width of the wound at × 100 magnification was evaluated by microscope. The length of the wound was measured at random intervals and the data were analyzed by ImageJ software.

16. Statistical analysis

Data are means ± standard deviation. Two-tailed unpaired Student's t tests were used to assess significance unless stated otherwise. P < 0.05 was deemed significant.

Results

1. Upregulation of E2F2 expression in GC

We first detected the expression of E2F2 in various tumor types. After analyzing more than a dozen cancers, including GC and their corresponding normal tissues, we found that the expression of E2F2

mRNA in GC tissues was significantly higher than that in paracancerous normal tissues (Figure 1A). We then used Oncomine and GEMIA to analyze the expression of E2F2 in GC tissues. The results showed E2F2 was significantly upregulated in GC tissues over normal gastric tissues (Figures B and C). The effect of E2F2 on the overall survival (OS) of GC patients was analyzed using K-M Plotter. We found that high E2F expression was associated with poor prognosis (Figure 1D). Further subgroup analysis of various clinicopathological features of TCGA-stomach adenocarcinoma (STAD) samples in the UALCAN database consistently showed that the E2F2 transcription level was elevated. In the subgroup analysis based on sex, age, race, disease stage, and tumor grade, the E2F2 expression in GC patients was significantly higher than that in the normal control group (Figure 2). Therefore, E2F2 expression might be a diagnostic indicator for GC.

2.The independent prognostic value of E2F mRNA expression in GC patients

We downloaded the clinical and E2F mRNA expression data of 407 GC patients from the TCGA database (Table 1). Survival analysis and Glmnet software were used to integrate and normalize the downloaded raw data, followed by univariate and multivariate survival analysis using Cox's regression model. In the univariate analysis, age, regional lymph node metastasis, distant metastasis, pathological stage, and E2F2 were closely correlated with the survival of patients (Table 2). In the multivariate analysis, the higher the pathological stage ($p = 0.036$) and the higher the E2F2 mRNA expression ($p = 0.005$), the shorter the OS of GC patients (Table 3).

3.Coexpression analysis and genomic alteration of E2F2 in GC

To better understand the biological significance of E2F2 in GC, we used the LinkedOmics functional module to study the coexpression patterns of E2F2 in the STAD cohort. As shown in Figure 3A, 9963 genes (dark-red dots) showed a positive correlation with E2F2, while 10,262 genes (dark-green dots) showed a negative correlation with E2F2 (false discovery rate <0.01). The heat map of Figure 3B shows the top 50 genes most strongly positively or negatively correlated with E2F2. Next, we used the cBioPortal tool to determine the types and frequencies of E2F2 changes in GC based on the DNA sequencing data of STAD patients. E2F2 was altered in 16 (3%) out of 473 patients with STAD (Figure 3C). There were five cases of missense mutations, five cases of high mRNA expression, four cases of amplification, and 11 cases of what cBioPortal calls deep deletion. As shown in Figure 3D, compared with the diploid group, the E2F2 expression level in the amplified group was higher. Figures 3E and 3F show the frequency distribution of E2F2 copy number variation (CNV) in groups with different stages and grades, suggesting that the incidence of E2F2 CNV changes was higher in GC and was an early event.

4.Relationship between E2F2 and immune cells and the programmed death-1/programmed death ligand-1 (PD-1/PD-L1)

We searched the TIMER database to assess the correlation between E2F2 mRNA expression and immune cell infiltration. As shown in Figure 4A, after the purity was adjusted, the E2F2 expression in GC was significantly associated with M1 cells (Figure 4A), M2 cells (Figure 4B), T helper (Th) 1 cells (Figure 4C),

Th2 cells (Figure 4D), and regulatory T (Treg) cells (Figure 4E). In addition, considering the prospect of immunotherapy, we further assessed the relationships between E2F2 expression and PD-1 and PD-L1 (Figure 4F). E2F2 was positively correlated with PD-1 ($r = 0.185$, $P = 1.49e-4$) and PD-L1 ($r = 0.279$, $P = 8.67e-9$). Normal tissues were used as the control to examine the effect of E2F2 on immune infiltration in different replication states using GISTIC2.0 data. We observed that E2F2 CNV was significantly associated with the degrees of infiltration of B cells, CD8⁺ T cells, CD4⁺ T cells, macrophages, neutrophils, and dendritic cells (Figure 4G).

5. Construction of the protein–protein interaction (PPI) network and gene enrichment analysis

To understand the function of E2F2 in GC, we searched the STRING website for proteins that interacted with E2F2. We screened the 50 genes with the highest correlations and used Cytoscape to construct a complete network (Figure 5A). We found that the genes associated with the cell cycle, including cyclin E1 (CCNE1), CCNE2, cyclin-dependent kinase 2 (CDK2), CDK4, CDKN1, and CDKN2A, were closely correlated with the changes in E2F2. Figure 5B shows 10 hub genes closely correlated with E2F2. Next, the functions of E2F2 and its 50 correlated genes were analyzed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. GO enrichment analysis predicts gene functions in three domains: biological process (BP), cellular component (CC), and molecular function (MF). As shown in Figure 5C, under BP, the E2F2-correlated genes were mainly enriched in biological regulation, metabolic processes, and response to stimuli. Under CC, the genes were mainly enriched in the membranes, nucleus, and membrane-coated lumens. The MFs were mainly protein binding, ion binding, and nucleic acid binding. KEGG enrichment analysis (Figure 5D) showed that these genes were the most abundant in cell cycle-related pathways, which was consistent with the results of the PPI analysis. The PI3K/Akt signaling pathway also exhibited a high degree of enrichment, so we studied this more.

6. E2F2 is a regulator of the PI3K/Akt/mTOR signaling pathway

Therefore, we hope to further validate the relationship between E2F2 and the PI3K/Akt signaling pathway through in vitro experiments. To confirm the high expression of E2F2 in GC tissues, immunohistochemical staining (IHC) was performed on 60 pairs of GC tissues and paracancerous tissues. Representative images are shown in Figure 6A. The E2F2 expression level in paracancerous normal tissues was significantly lower than that in cancer tissues. We performed qPCR and western blotting on matched GC and adjacent nontumor frozen tissues. We observed that E2F2 was significantly overexpressed in GC (Figure 6B and 6C). The E2F2 expression in cultured GC cells was detected by qPCR and western blotting. We found that E2F2 mRNA and protein were significantly upregulated in AGS and HGC27 cells compared with GES-1 cells (Figures 6D and 6E). To better understand the impact of E2F2 on the biological behavior of GC, we used GV141-E2F2 and siE2F2 to transfect HGC27 cells and AGS cells, respectively. High overexpression efficiency and gene knockout efficiency were observed in the treated cells (Figure 6F and G). Subsequently, we investigated the effect of E2F2 overexpression and knockdown on the PI3K/Akt/mTOR pathway in these cell lines. Western blotting showed that at 24 h after forced E2F2 overexpression, PI3K110 expression, phosphorylated Akt (p-Akt), and phosphorylated mTOR (p-mTOR)

were increased in HGC27 cells (Figure 6H). In contrast, after E2F2 was knocked down by siE2F2 for 24 h, the expression levels of PI3K110 β , p-Akt, and p-mTOR were significantly decreased in AGS cells (Figure 6I).

7.Effects of autophagy-mediated E2F2 expression on the migration and invasion of GC cells

Since E2F2 can regulate the PI3K/Akt/mTOR pathway and the PI3K/Akt/mTOR pathway plays an important role in the process of autophagy (28,29), we further studied the relationship between E2F2 and the expression of autophagy-related proteins by transfecting GC cells with GV141-EE2F2 or siE2F2. We found that after E2F2 overexpression, LC3II expression decreased significantly, while p62 protein was significantly increased (Figure 7A). In contrast, after E2F2 inhibition, LC3II increased significantly, while p62 protein was significantly decreased (Figure 7B). Interestingly, in HGC27 cells transfected with GV141-E2F2, the number of autophagic vesicles also significantly decreased, while the number of autophagic vesicles was the highest in AGS cells transfected with siE2F2 (Figure 7C). In summary, our data showed that E2F2 overexpression inhibited autophagy in GC cells and inhibited E2F2-induced autophagy. We observed that E2F2 overexpression significantly increased the migration and invasion of GC cells (Figure 7D and 7E). When cells were cotreated with siE2F2 and the autophagy inhibitor 3-MA, the inhibition of the E2F2-mediated antimetastatic effect was reversed (Figure 7F and 7G). This combined treatment significantly reduced the expression of the E2F2 inhibition-dependent LC3-II and Beclin1 proteins and increased the protein expression of p62 and matrix metalloproteinase 9 (MMP9) (Figure 7H). Therefore, our results suggest that E2F2 overexpression promotes the migration and invasion of GC cells by inhibiting autophagy. Inhibition of E2F2 induces autophagy, thereby inhibiting the migration and invasion of GC cells.

Discussion

E2F2 plays contradictory roles in tumor occurrence and development. On the one hand, E2F2 inhibits tumorigenesis by inhibiting cell cycle regulation. On the other hand, E2F2 can act to upregulate target genes, leading to cancer (30). Changes in E2F2 protein expression are closely related to the occurrence of different cancers. Knockout of the E2F2 gene significantly reduced the metastasis ability of breast cancer cells, and E2F2 gene mutation was associated with tumor proliferation and survival in breast cancer patients (11). E2F2 plays an oncogenic role in liver cancer (10), whereas in prostate cancer, E2F2 inhibits tumor cell proliferation by targeting microRNAs (miRNAs) (31). The role of E2F2 in GC has not been fully confirmed. To understand the potential function of E2F2 in GC and its regulatory network in more detail, we conducted bioinformatic analysis on published data followed this with experimental validation to guide future studies on GC.

Using a large number of independent datasets, we confirmed that E2F2 expression was significantly increased in GC tissues compared with nontumor gastric tissues. The prognosis of GC patients with high E2F2 expression was worse than that of patients with low E2F2 expression. Consistent with this, our Cox regression model confirmed that the E2F2 expression level was an independent predictive factor for the

OS of GC patients. Subgroup analysis was performed on multiple clinicopathological features of TCGA-STAD specimens. Subgroup analysis based on sex, age, race, disease stage, and tumor grade showed that E2F2 expression in GC patients was significantly higher than that in the normal control group. Therefore, our results indicate that E2F2 is upregulated in many GC cases, and as a potential diagnostic and prognostic marker, it is worth further clinical validation.

A recent study found that genomic alterations, such as somatic mutations in the genes encoding spliceosome components, often occur in human tumors (32). CNV may have significant genomic implications, such as interfering with the gene expression, altering the genetic content, and causing phenotypic differences (33). Our study found that E2F2 gene copy number was increased in GC tissues, and the major types of E2F2 gene change were missense mutation and amplification, which were associated with shorter survival time.

The tumor microenvironment refers to nontumor cells that exist inside and around a tumor. It has a great influence on the genomic analysis of tumor samples (34). Tumor-infiltrating immune cells are part of the complex microenvironment and are associated with the biological behavior of GC and the survival of patients (35). In this study, we observed that E2F2 expression in GC tissues was positively correlated with the infiltration of M1/M2 cells, Th1/Th2 cells, Tregs, and macrophages. E2F2 regulates the sensitivity of cells to external signals by negatively regulating the AhR pathway of T lymphocytes (36). Our results support the specific correlation between E2F2 and infiltrating immune cells in GC, suggesting that E2F2 plays an important role in immune infiltration into the tumor microenvironment. Whether E2F2 is a key factor in the mediation of immunotherapy requires further study.

The observed correlation between E2F2 expression and the mTOR pathway (from the KEGG analysis) enabled us to study the novel role of E2F2 in autophagy, and this role was regulated by the PI3K/Akt/mTOR pathway. Specifically, our data showed that E2F2 overexpression upregulated the PI3K/Akt/mTOR pathway, resulting in inhibition of autophagy and promotion of the migration and invasion of GC cells in vitro. E2F2 inhibition downregulated PI3K/Akt/mTOR activity, resulting in autophagy and inhibition of GC cell migration and invasion. The autophagy inhibitor 3-MA partially reversed the inhibitory effect of E2F2 on GC cell migration and invasion, further confirming the contribution of autophagy to these processes. Previous studies have reported the role of E2F1 in autophagy regulation (37). E2F2, as a member of the E2F transcription factor family, has no confirmed role in autophagy. Our study is the first to find that E2F2 may play an important role in autophagy. This may provide novel insights into the dual role of E2F2 in cancer cells. E2F2 may regulate the growth and progression of GC in complex ways, which may be achieved through the regulation of autophagy. Autophagy has been proven to be a double-edged sword in different cancers, including GC.

Conclusion

This study provides multilevel evidence for the significance of E2F2 in the occurrence of GC and its potential as a biomarker in GC. E2F2 was overexpressed in GC, and high E2F2 expression was associated

with the characteristics of invasive tumors and poor patient prognosis. The levels of immune infiltration and a series of immunological markers were significantly correlated with E2F2 expression in GC. Our findings provide novel insights into the role of E2F2 in prognosis and the potential role of E2F2 in GC tumor immunology. In terms of function, we discovered a novel mechanism by which E2F2 regulated autophagy, at least in part through the PI3K/Akt/mTOR pathway, which in turn regulated the migration and invasion of GC cells. These findings need to be confirmed by large-scale clinical studies and more in-depth mechanistic studies. The current results are encouraging, as our findings may provide novel ideas and strategies for the diagnosis and treatment of GC.

Abbreviations

GC: gastric cancer; TIMER: Tumor Immune Estimation Resource; DAVID: Database for Annotation, Visualization, and Integrated Discovery; OS: overall survival; PI3K: phosphoinositide-3 kinase; mTOR: mammalian target of rapamycin; TCGA: The Cancer Genome Atlas; GEPIA: Gene Expression Profiling Interactive Analysis; GO: gene ontology ; KEGG: Kyoto Encyclopedia of Genes and Genomes; BPs: biological processes; CC: cell composition ; MF: molecular function; TMAs: Tissue microarrays; IHC: Immunohistochemistry; FBS: fetal bovine serum; qPCR: quantitative real-time PCR; PBS: phosphate-buffered saline; CNV: copy number variation

Declarations

Ethics approval and consent to participate

Collection of samples used in this study was approved by the Ethics Committee of The Affiliated Hospital of Qingdao University

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article. Further details are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

HL and SZ analyzed the data and wrote the manuscript. LS, PW and SL assisted in editing the manuscript. YM, ZL and GW contributed to the design of the study. WQ and JL are the corresponding authors of the paper. All authors read and approved the final manuscript.

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Tables

Table 1 Basic characteristics of 366 GC patients.

Variables	GC patients(N=366)
Gender(Male/female)	235/131
Age(years, Mean±SD)	65.57±10.27
Tumor	
T1	17
T2	81
T3	169
T4	98
Regional lymph node	
N0	114
N1	97
N2	78
N3	70
Metastasis	
M0	325
M1	24
Histologic grade	
1	8
2	129
3	220
Pathologic stage	
1	51
2	124
3	151
4	39

Table 2 Univariate analysis of overall survival in 366 GC specimens.

Variables	Univariate analysis		
	Hazard Ratio	95% CI	P value
Gender	0.775	0.544-1.105	0.160
Age(years)	1.022	1.005-1.039	0.011
Tumor			0.109
T(1)	0.098	0.013-0.710	0.022
T(2)	0.768	0.480-1.229	0.271
T(3)	0.882	0.601-1.294	0.521
Regional lymph node			0.008
N(1)	0.981	0.134-7.187	0.985
N(2)	1.328	0.182-9.687	0.780
N(3)	1.626	0.222-11.917	0.632
Metastasis			0.023
M(1)	0.575	0.282-1.453	0.154
Histologic Grade			0.157
G(1)	0.284	0.030-2.734	0.276
G(2)	0.711	0.221-2.293	0.568
Pathologic Stage			0.002
S(1)	0.134	0.032-0.571	0.007
S(2)	0.391	0.191-0.803	0.011
S(3)	0.424	0.202-0.892	0.024
E2F2	0.885	0.792-0.988	0.030

Table3 Multivariate analysis of overall survival in 366 GC specimens.

Variables	Multivariate analysis		
	Hazard Ratio	95% CI	P value
Age(years)	1.033	1.014-1.052	0.001
Regional lymph node			0.300
N(1)	2.002	0.245-16.384	0.518
N(2)	1.459	0.190-11.209	0.716
N(3)	2.004	0.250-16.034	0.512
Metastasis			0.529
M(1)	0.631	0.261-1.526	0.307
Pathologic stage			0.036
S(1)	0.114	0.021-0.617	0.012
S(2)	0.29	0.093-0.902	0.033
S(3)	0.439	0.160-1.206	0.010
E2F2	0.841	0.745-0.950	0.005

Figures

Figure 1

The expression and methylation of E2F2 in GC tissues and normal tissues revealed by bioinformatic analysis. (A) E2F2 expression levels of different tumor types in the TCGA database were detected by TIMER (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (B and C) E2F2 mRNA is highly expressed in LGG tissues in GEPIA dataset (B) and Oncomine dataset. (D) Kaplan-Meier analysis of survival rates of GC patients with high E2F2 expression and GC patients with low E2F2 expression.

Figure 2

E2F2 transcription in subgroups of patients with GC, stratified based on gender, age and other criteria (UALCAN). (A) Boxplot showing relative expression of E2F2 in normal and STAD samples. (B) Boxplot showing relative expression of E2F2 in normal individuals of any age or in STAD patients aged 21-40, 41-60, 61-80, or 81-100 yr. (C) Boxplot showing relative expression of E2F2 in normal individuals of either gender and male or female STAD patients, respectively. (D) Boxplot showing relative expression of E2F2 in normal individuals or in STAD patients in stages 1, 2, 3 or 4. (E) Boxplot showing relative expression of E2F2 in normal individuals or STAD patients with grade 1, 2, 3 or 4 tumors. (F) Boxplot showing relative expression of E2F2 based on TP53 mutation status. The central mark is the median; the edges of the box are the 25th and 75th percentiles. The t-test was used to estimate the significance of difference in gene expression levels between groups. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Figure 3

E2F2 co-expression genes (LinkedOmics) and genomic alterations (cBioPortal) in GC. (A) The global E2F2 highly correlated genes identified by Pearson test in STAD cohort. (B) Heat maps showing top 50 genes positively and negatively correlated with E2F2 in STAD. Red indicates positively correlated genes and blue indicates negatively correlated genes. (C) OncoPrint of E2F2 alterations in STAD cohort. The different types of genetic alterations are highlighted in different colors. (D) E2F2 expression in different E2F2 CNV groups. (E and F) Distribution of E2F2 CNV frequency in different stage and grade subgroups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4

The expression of E2F2 was related to a panel of gene markers of immune cells, including M1 cell (A), M2 cell (B), Th1 cell (C), Th2 cell (D), Treg cell (E) and PD-1/PD-L1 (F). (G) E2F2 CNV affects the infiltrating levels of B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils and dendritic cells in GC

list and in the category. (D) Pathways enrichment map of E2F2 and its 50 frequently altered neighbor genes. The top 20 terms with the largest number of enriched genes were selected.

Figure 6

E2F2 is a regulator of the PI3K/Akt/mTOR pathway. (A) Representative images of E2F2 TMA analysis in GC tissues and adjacent tissues. Scale bar, 250μm. (B) Quantitative PCR analysis of E2F2 mRNA expression in GC and non-tumor gastric tissues in our patient cohort. E2F2 mRNA expression levels were

normalized according to glyceraldehyde 3-phosphate dehydrogenase expression levels (n = 20 per group). (C) Western blotting analysis of E2F2 protein expression in GC (T) and non-tumor gastric tissues (N). E2F2 protein expression levels were normalized according to β -actin expression levels (n = 8 per group). (D) Quantitative PCR (qPCR) analysis of E2F2 basal mRNA expression in three cell lines. E2F2 mRNA expression levels were normalized according to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels. (E) Western blotting analysis of E2F2 basal protein expression in the three cell lines; β -actin was used as a loading control. (F) Western blotting analysis of E2F2 protein expression in HGC27 cells transfected with GV141-Vector and GV141-E2F2 for 24 h. β -Actin was used as a loading control. (G) Western blotting analysis of E2F2 protein expression in AGS cells transfected with siE2F2 for 24 h. β -Actin was used as a loading control. (H) Western blotting analysis of the PI3K 110 β , p-AKT, AKT, p-mTOR, and mTOR protein expression in HGC27 cells transfected with GV141-Vector and GV141-E2F2 for 24 h. β -Actin was used as a loading control. (I) Western blotting analysis of PI3K 110 β , p-AKT, AKT, p-mTOR and mTOR protein expression in AGS cells transfected with siNC and siE2F2 for 24 h. β -Actin was used as a loading control. Data are presented as mean $\pm$ S.D. from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7

Effects of E2F2 expression levels on GC cell migration and invasion via autophagy mediation. (A) Western blotting analysis of P62, and LC3-II protein expression in GC cells transfected with GV141-Vector and GV141-E2F2. β -Actin was used as a loading control. (B) Western blotting analysis of P62, and LC3-II protein expression in GC cells transfected with siNC and siE2F2. β -Actin was used as a loading control. (C) Representative electron micrographs of autophagic vesicles in GC cells transfected with GV141-Vector

and GV141-E2F2 and in GC cells transfected with siNC and siE2F2. (D) GC cells were transfected with GV141-Vector and GV141-E2F2 for 24 h. Comparison of the migration and invasion GC cells using transwell compartments. (E) Wound-healing assay comparing the motility of GC cells. The wound-healing area was analyzed using the ImageJ software. (F) GC cells were transfected with siNC and siE2F2 or treated with phosphate-buffered saline (control) or 3-methyladenine (2 mM) or a combination of both treatments for 24 h. Comparison of the migration and invasion of GC cells using transwell compartments. (G) Wound-healing assay comparing the motility of GC cells. (h) Western blotting analysis of P62, Beclin1, LC3-II and MMP9 protein expression. β -Actin was used as a loading control. Data are presented as mean $\pm$ S.D. from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Supplementary Files

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