Original Article

E2F1/2/4 mRNA is associated with immune infiltration and are potential biomarkers for the prognosis of human gastric carcinoma

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Background: E2Fs are genes that regulate DNA synthesis and the cell cycle by encoding a family of transcription factors. Increasing experimental evidence has revealed that E2Fs play key roles in tumor progression in various types of cancer.

Methods: We investigated the survival, expression and transcriptional data of E2F1/2/4 in gastric cancer (GC) patients using the immunohistochemistry assay, Kaplan-Meier Plotter, cBioPortal, String, and GEPIA databases. The plasma of GC patients was analyzed using the real-time reverse transcription polymerase chain reaction (RT-PCR) assay. The correlation between E2F1/2/4 expression and clinical features was analyzed using the quartile method. As well, the correlation between E2F1/2/4 and GC immune infiltration was also investigated using the TIMER database. Database of Immune Cell Expression (DICE) was also used to analyze correlations between SOX4 and immune responses.

Results: RT-PCR and tissue immunohistochemistry confirmed that E2F1/2/4 was highly expressed in serum and GC tissue samples of GC patients, the expression of which was not affected by patient age and gender. Also, the survival analysis revealed that low levels of E2F1/2/4 expression were significantly associated with a longer overall survival (OS) in GC patients. E2F1/2/4 was correlated with patient prognosis and immune cell infiltration, including B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and DCs in GC. Our findings indicated that E2F1/2/4 could be used as a prognostic biomarker and indicator of immune infiltration in GC.

Conclusions: This study revealed that E2F1/2/4 could be a promising indicator for tumor-associated immune infiltration and prognosis in GC patients.

Keywords: E2F1/2/4; gastric cancer (GC); immune infiltration; bioinformatics analysis

Submitted Jan 08, 2021. Accepted for publication Apr 27, 2021.
doi: 10.21037/tcr-21-45
View this article at: https://dx.doi.org/10.21037/tcr-21-45

Introduction

Gastric cancer (GC) is the third leading cause of cancer-related death worldwide and the fifth most commonly diagnosed cancer (1). GC pathogenesis is a multistep and multifactorial process involving tumor gene mutations and epigenetic changes (2). Currently, although conventional tumor biomarkers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) have been widely

used in the diagnosis of GC, they cannot be applied in the screening of early GC due to their shortage in terms of specificity and sensitivity (3). With the increasing studies on carcinogenesis in recent years, it has become increasingly important to search for indicators affecting the development and prognosis of GC at the molecular level and to develop targeted therapies. Currently, there are more and more drugs and studies targeting human epidermal growth factor receptor-2 (HER-2), vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), serine/threonine-protein kinase mTOR, immune checkpoints and other related pathways and targets, as well as certain breakthroughs have been achieved, but most of phase III studies of targeted therapies for GC, such as the EXPAND trial of cetuximab and GRANITE-1 trial of everolimus, showed a negative result (4-6). Currently, only angiogenesis and HER2 are the most promising and attractive targets in the treatment of GC, and the 5-year overall survival (OS) rate still remains poor (4,7). Therefore, further exploration and research are needed in the fields of etiology and molecular diagnostics of GC, and new biomarkers with high specificity that can be detected early and lead to treatment options are developed. Thus, there is a need for potential prognostic biomarkers and drug targets for GC.

In mammalian cells, E2Fs are a series of genes encoding transcription factors (8). E2Fs can be divided into 2 groups according to their functions. E2F1, E2F2, and E2F3a function as transcriptional activators (9); whereas E2F4-8 and E2F3b act as transcriptional repressors (10). Moreover, E2Fs regulate a series of biological effects, including DNA synthesis, gene expression and cell cycle regulation (11). Moreover, E2Fs are closely related to various cancers' progression and are widely expressed in different types of cancer tissues (12).

Previous studies have identified a total of E2F1/2/3/4/5/6/7/8 factors in mammalian cells (13). A recent study identified that E2F1, E2F2, E2F3, and E2F8 function as oncogenes in lung cancer (14). E2F1 plays a conflicting role in different types of cancers as it exhibits tumor-suppressing activity in gastric, esophageal, and colorectal cancer, but displays the opposite function in pancreatic ductal adenocarcinoma and esophageal squamous cell carcinoma (15). E2F1 also affects the epithelial to mesenchymal transition to promote the progression of GC. A recent study also indicated that E2F6 promotes gastric carcinoma progression by downregulating lncRNA CASC2 (16). However, little is known about the features and prognosis induced by an abnormal expression of E2F1/2/4 expression in GC (17). Clinical performance of E2Fs 1–3 in kidney clear cell renal cancer, evidence using a bioinformatics analysis (18). Also, a previous study analyzed the expression and mutations of E2Fs in GC (19). Based on these analyses, E2Fs were found to be correlated with infiltrating immune cells level, such as $CD4^+$ T cells, $CD8^+$ T cells, and DCs in GC patients. Our results suggested that E2F1/2/4 functioned as biomarkers for determining the prognosis and immune infiltration in GC.

We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/tcr-21-45).

Methods

GEPIA analysis

The transcription levels of E2Fs in GCs was analyzed in an online cancer microarray database GEPIA database (www.oncomine.org).

Survival analysis

Kaplan-Meier Plotter online database was used to evaluate the prognostic value of E2FS in GC patients as previously reported. Data of gene expression and patient survival were obtained from TCGA database (http://www.tcga.org/) and the Kaplan-Meier plotter (http://kmplot.com/), respectively. The details of data analysis were as previously described (20,21).

Database of immune cell expression (DICE)

DICE (https://dice-database.org/landing) was used to analyze the expression of E2F1/2/4 in various immune cells.

TIMER and STRING database analysis

TIMER (https://cistrome.shinyapps.io/timer/) was used to estimate immune infiltrates. In this study, E2FS expression have correlation with immune infiltrates, including B cells, $CD4^+$ T cells, $CD8^+$ T cells. The related marker genes on the y axis as gene symbols, the E2FS was used for the x axis with gene symbols. The expression level of gene was displayed with log2 RSEM. SCNAs in TIMER database are defined by GISTIC 2.0. Protein-protein interactions were predicted by STRING database (https://string-db.org/cgi/about?footer_active_subpage=content).

The Cancer Genome Atlas (TCGA) data and cBioPortal

TCGA including 30 different kinds of cancer types, and also had both sequencing and pathological data (22). cBioPortal (http://www.cbioportal.org/index.do?session_id=5a37ba8e498eb8b3d56242fb) was used to analyses of E2Fs in the stomach adenocarcinoma (TCGA, Provisional) dataset. cBioPortal's online instruction was used to calculate E2Fs network and co-expression.

Tissue immunohistochemistry for E2F1 and E2F2 expression

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Tumor tissues and corresponding non-tumor-adjacent tissues of GC patients were selected in Biobank of Zhejiang Cancer Hospital. The clinical features of the patient including age, gender, tumor stage, CEA and CA19-9 expression were derived from what was registered in our biobank. The Medical Ethics Committee, Zhejiang Medical College approved our study (No. IRB -2020-407). The immunohistochemical staining kit (PV9001, Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.) was used for this study. E2F1 (No. sc-56661, Santa Cruz) and E2F2 antibody (No. Sc-9967, Santa Cruz) (1:100) was added to each section. Considering the retrospective design of our study, the informed consent was waived.

Quantitative real-time PCR

Mini Kit (Qiagen) was used for total RNA extracted. The primers for E2F1/2 and U6 were synthesized by Sangon Biotech (Shanghai, China). The primers for U6 were: 5'-CTCGCTTCGGCAGCACATATACT-3' and 5'-ACGCTTCACGAATTTGCGTGTC-3'. The primers for E2F1 were: 5'-GCCACTGACTCTGCCACCATAG-3' and 5'-CTGCCCATCCGGGACAAC-3'. The primers for E2F2 were: 5'-CCTTGGAGGCTACTGACAGC-3' and 5'-CCACAGGTAGTCGTCCTGGT-3'. The primers for E2F4 were: 5'-GACCCCACAGGTGTTTTG-3' and 5'-CCAGGTTGTAGATGTAATCG-3'.

Statistical analysis

Graphpad Prism software (Version 7.0) was used for statistical analysis. Patients were divided into two groups according to E2F1/2/4 expression (high *vs.* low) in Kaplan-Meier Plotter online database. Kaplan-Meier survival plots were used to compare OS between the two groups. Hazard ratio (HR) values with 95% CI and log-rank P values were calculated. The correlation between E2F1/2/4 expression and clinical features was analyzed using the quartile method. The differences between groups were analyzed by the Students' *t*-test. P<0.05 was considered statistically significant.

Results

Relationship between GC and the expression of E2Fs

The GEPIA dataset was used to analyze the differences in the levels of E2F1/2/3/4/5/6/7 expression between GC and normal stomach tissues (*Figure 1A*,*B*). According to the results, E2F1, E2F2, E2F4, E2F5, and E2F6 were highly expressed in stomach adenocarcinoma compared with normal stomach tissues.

E2F1/2/4 were up-regulated in patients with GC

To confirm the expression of E2F1/2/4, we analyzed serum expression levels of E2F1, E2F2, CEA, and CA19-9. As shown in *Figure 2A* showed, E2F1/2/4 were highly expressed in the serum of GC patients, and had higher sensitivity and specificity than CEA and CA19-9. The details were descripted in *Table 1*. Furthermore, gastric tumor tissues and corresponding adjacent non-tumor gastric tissues (10 pairs) were used to confirm E2F1 and E2F2 expression. IHC staining showed abundant and uniform expression of E2F1/2 proteins in tumor samples (*Figure 2B*). Additionally, the expression levels of E2F1, E2F2 and E2F4 were numerically higher in patients with advanced stages (stage III or IV) compared to those with stage I or II, with a statistically significant difference in E2F4 expression between the two groups (P<0.05, *Table 2*).

Prediction of pathways and functions based on changes in E2F1/2/4

The cBioPortal online tool was used to analyze the alterations, correlations, and networks associated with E2Fs. E2Fs were found to be altered in 328 samples out of 634 patients with GC (40%). Additionally, out of 164 samples, in approximately half of the samples, 2 or more alterations were detected (*Figure 3A*,*B*). Furthermore, we explored E2F mRNA expression (RNA Seq V2 RSEM). The online

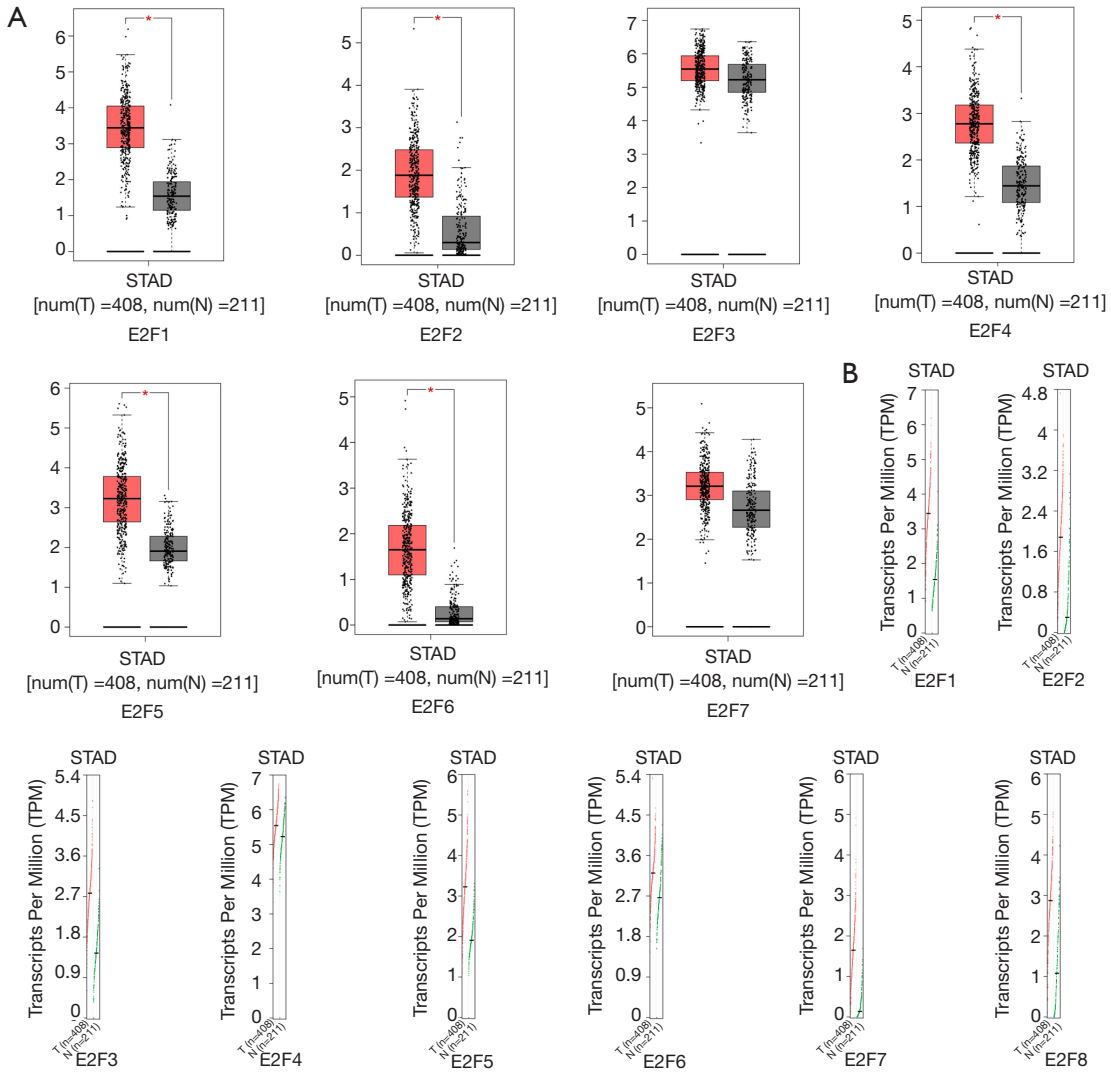

Figure 1 The expression of E2Fs in gastric cancer (GEPIA). (A,B) The higher expression levels of E2F1/2/4/5/6 in GC patients were represented as bar graphs and scatter plots, respectively. GC, gastric cancer. *, P<0.05.

cBioPortal tool was used to calculate the correlations among E2F1/2/4/5/7/8 in GC (TCGA, Provisional), including Pearson's correlation coefficient. This analysis revealed that E2F1 with E2F2, E2F2 with E2F1, and E2F7 and E2F8 showed significant positive correlations (*Figure 3C*). According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases, the functions of E2Fs and their associated genes were concentrated in the cell cycle, the p53 signaling pathway, and cellular senescence regulation (*Figure 3D,E,F,G*). The E2Fs were correlated with well-established genes and pathways involved in various cell cycle processes.

Expression of E2F1/2/4 in various types of immune cells

We further constructed the frequently altered E2F1/2/4 neighboring gene regulatory network, which revealed that E2F1/2/4 was highly associated with cell cycle genes, such as CDK4 and CDK6 CCNE1, and CDKN2A (*Figure 4A*). A recent study indicated that CDK4/6 could control the production of neutrophil extracellular traps. Also, E2F1/2 could determine the threshold for antigen-induced T cell proliferation (12). Thus, we sought to explore the expression of E2F1, E2F2, and E2F4 in various immune cells. According to the immune cell database, E2F1/2 exhibited high expression in $CD4^+$ T cells and macrophages

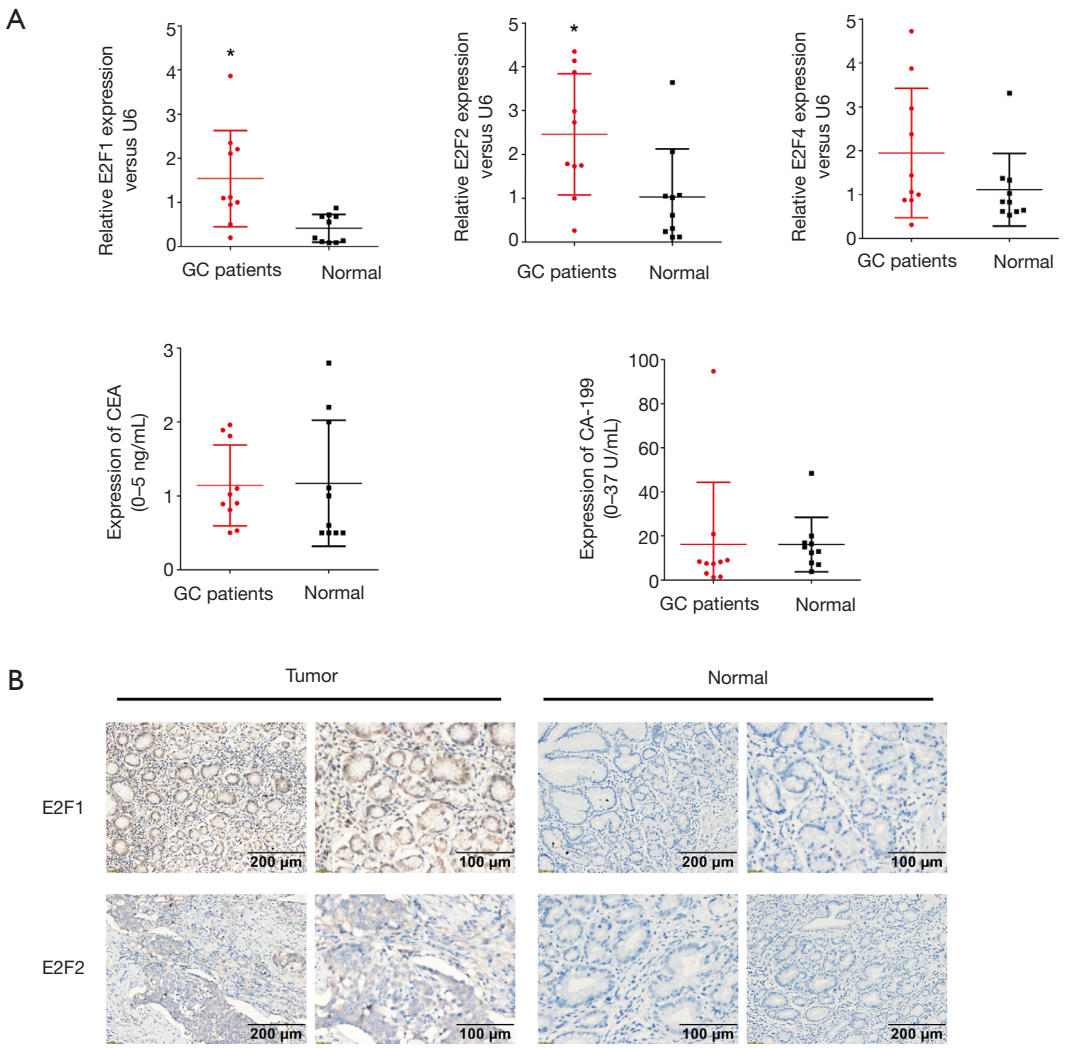

Figure 2 High expression levels of E2F1/2/4 in GC. (A) High expression levels of E2F1/2/4 in serum from patients with GC (n=10) compared to healthy individuals (n=10). Expression levels were relative to control U6 expression. (B) E2F1/2 immunohistochemistry in gastric tumor and adjacent non-tumor gastric tissue. GC, gastric cancer. *, P<0.05.

Table 1 Clinical features in gastric cancer patients (gastric) and healthy individuals (control)

Groups	Number	Age (years)	Male	CEA (P_{25}, P_{75})	CA19-9 (P_{25}, P_{75})	E2F1 (P_{25}, P_{75})	E2F2 (P_{25}, P_{75})	E2F4 (P_{25}, P_{75})
Gastric	10	51.5 (45, 61.5)	40%	0.96 (0.74, 1.83)	7.83 (2.56, 11.94)	1.1 (0.83, 2.23)	2.25 (1.54, 3.93)	1.25 (0.87, 3.19)
Control	10	46 (31.7, 53.5)	40%	0.8 (0.5, 2.0)	13.98 (7.61, 17.59)	0.373 (0.10, 0.68)	0.81 (0.21, 1.31)	0.83 (0.61, 1.33)

Table 2 Correlation between serum E2F1/2/4 expression and tumor stage in patients with gastric cancer

		E2F1 (P_{25}, P_{75})	E2F2 (P_{25}, P_{75})	E2F4 (P_{25}, P_{75})	CEA (P_{25}, P_{75})	CA19-9 (P_{25}, P_{75})
UICC stage	I+II	1.02 (0.61, 2.03)	1.76 (0.63, 3.70)	0.87* (0.45, 1.01)	0.67 (0.50, 1.67)	7.41 (2.73, 8.01)
	III+IV	1.61 (0.79, 2.61)	2.85 (1.54, 3.93)	2.67 (1.32, 4.08)	1.06 (0.89, 1.83)	8.63 (2.56, 39.27)

UICC, Union for International Cancer Control; *, P<0.05.

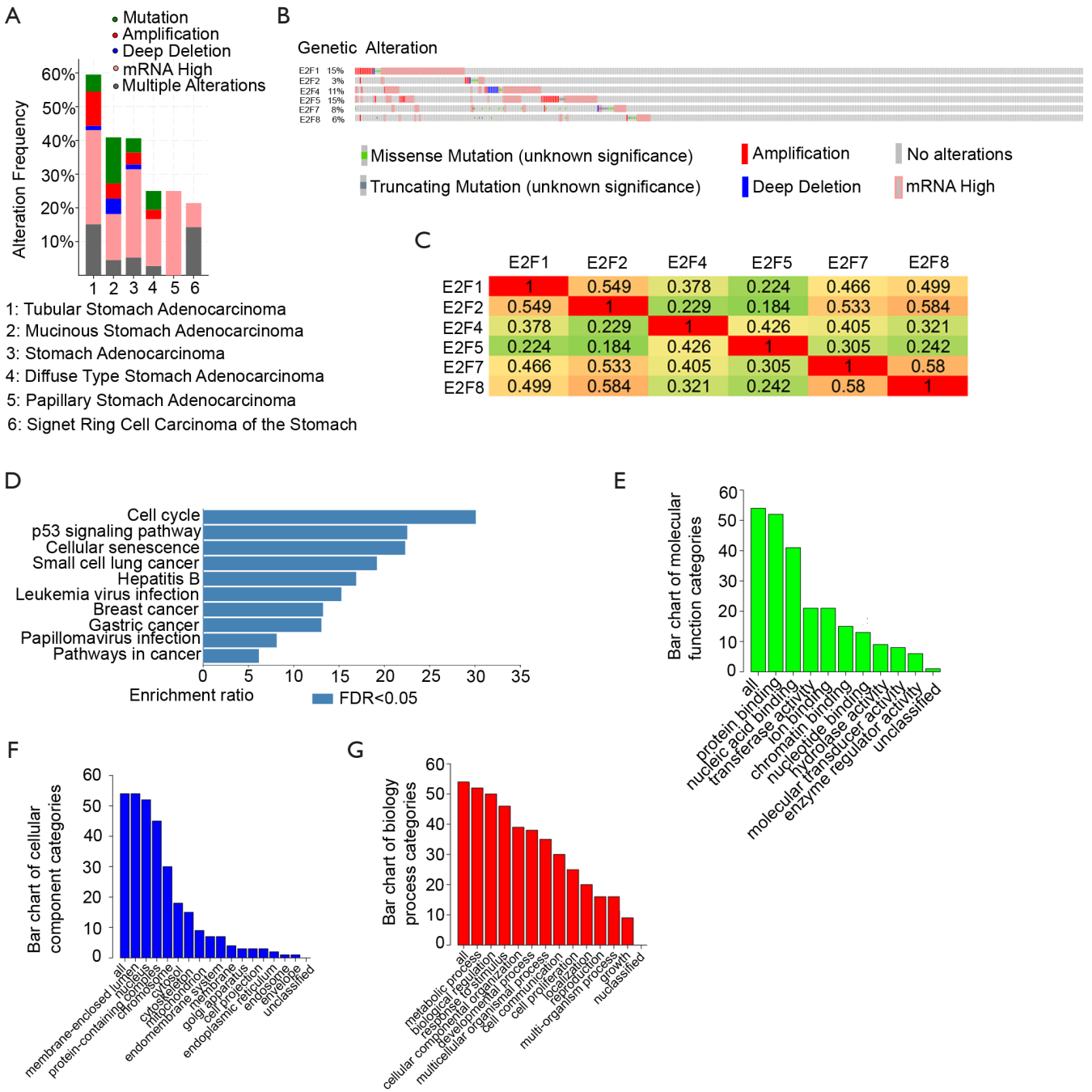

Figure 3 E2Fs mutation analysis and functions in GC. (A,B) Mutation analysis of E2Fs. (C) The interactions between E2F1/2/4/5/7/8. (D,E,F,G) GO analysis of the E2F-associated functions and pathways in GC. GC, gastric cancer; GO, Gene Ontology.

(*Figure 4B,C*), whereas E2F4 was highly expressed in CD4$^+$ T cells and CD8$^+$ T cells (*Figure 4D*). These results suggested that the expression of E2F1/2/4 was closely correlated with CD4$^+$ T cells.

E2F1/2/4 expression is correlated with the level of immune infiltration in GC

Furthermore, we explored the association between E2F1/2/4 and the level of immune infiltration and patient prognosis

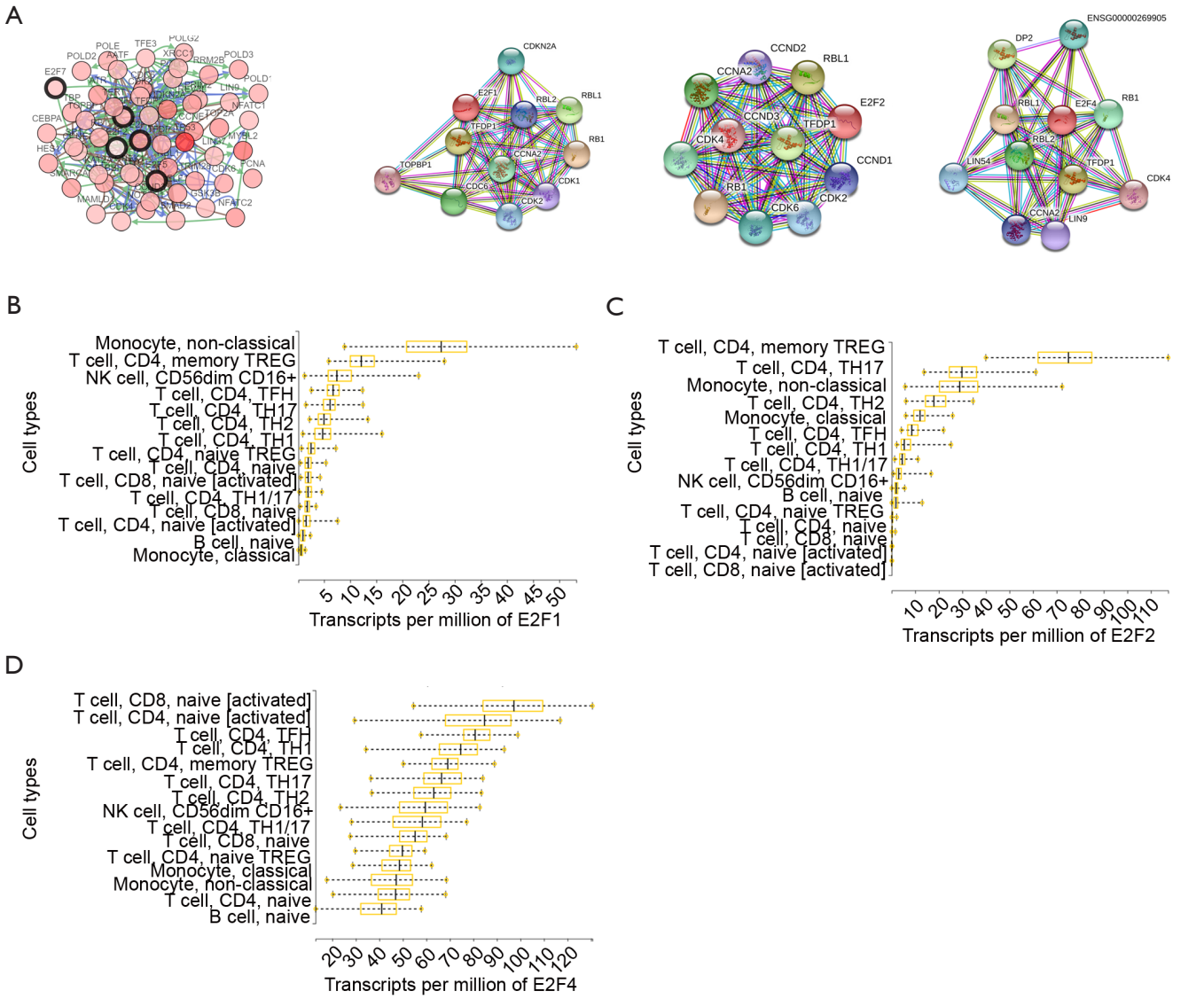

Figure 4 Bioinformatics analysis of the regulatory network and expression of E2F1/2/4 in immune cells. (A) E2F1/2/4 interaction with CDK2/4/6. (B) E2F1 is highly expressed in monocyte cells and T cell CD4 memory TREGs. (C) E2F2 is highly expressed in CD4 T memory TREGs and Th17 cells. (D) E2F4 is highly expressed in both CD4 and CD8 T cells.

in GC (23). Immune infiltration in clinical tumor samples using the genomic approach and the TIMER database was influenced by tumor purity (24-26). E2F1/2/4 was found to have a significant positive correlation with GC tumor purity (*Figure 5A*). Interestingly, the level of E2F1/2/4 expression was negatively correlated with infiltrating levels of $CD8^+$ T cells, $CD4^+$ T cells, macrophages, B cells, neutrophils, and DCs in GC (*Figure 5B*). These results suggested that E2F1/2/4 played a specific role in immune infiltration in GC and suppressed tumorigenesis.

E2F1/2/4 were significantly associated with the OS of patients with GC

We further investigated the critical efficiency of E2Fs for the prognosis of GC patients. Kaplan-Meier Plotter tools (http://kmplot.com/) were used to analyze the correlation between the level of E2F1/2/4 mRNA and GC patient survival. Low E2F1/2/4 expression was highly correlated with a longer OS (all P<0.05; *Figure 6A,B,C*). These findings suggested that E2F1/2/4 might have the potential to predict

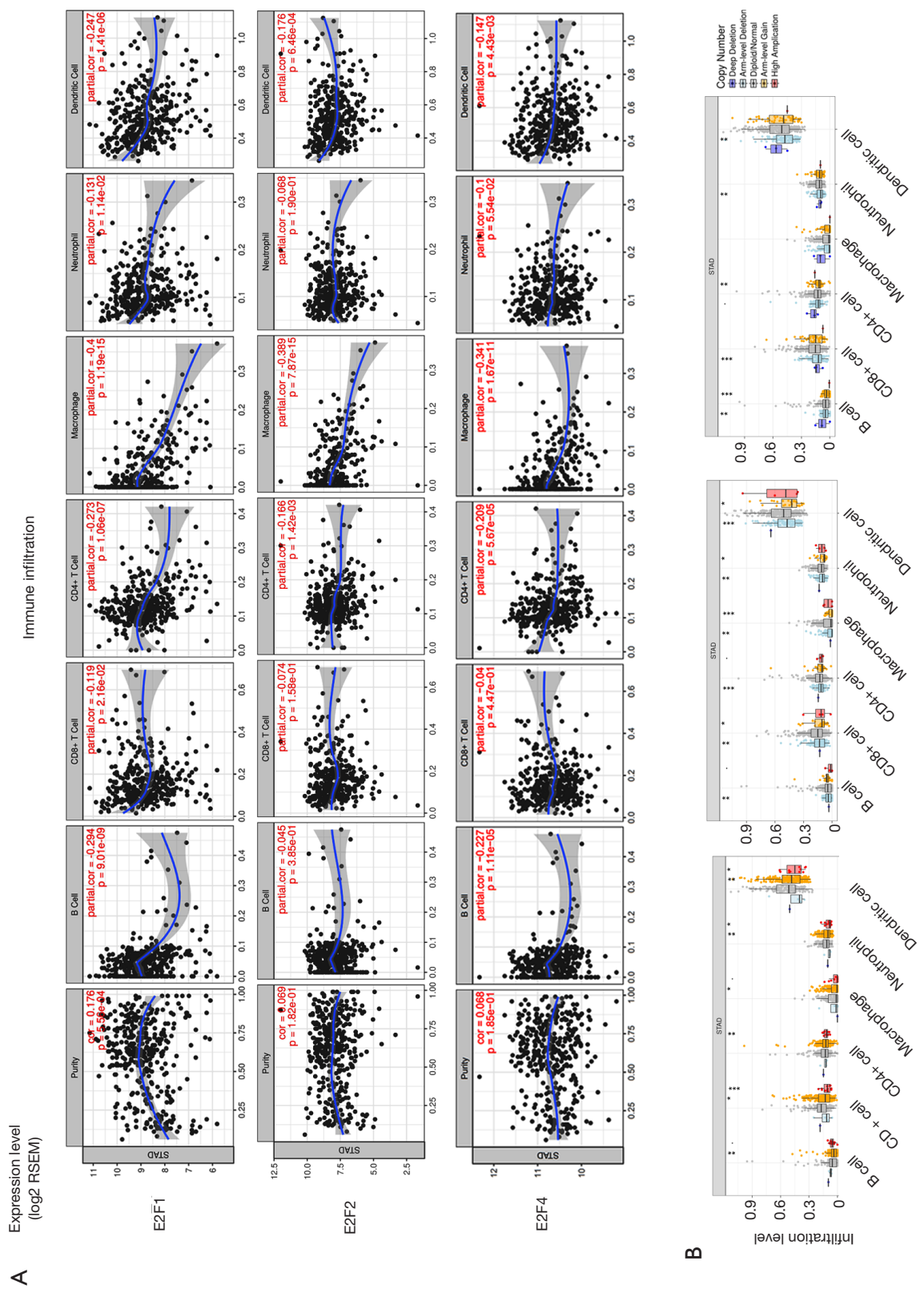

Figure 5 Correlation of E2F1/2/4 expression with the level of immune infiltration in STAD. (A) The expression of E2F1/2/4 was significantly negatively correlated with the levels of infiltrating $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells and was positively related to tumor purity. (B) The infiltration level of different somatic copy number alterations for E2F1/2/4. *, P<0.05; **, P<0.01; ***, P<0.001.

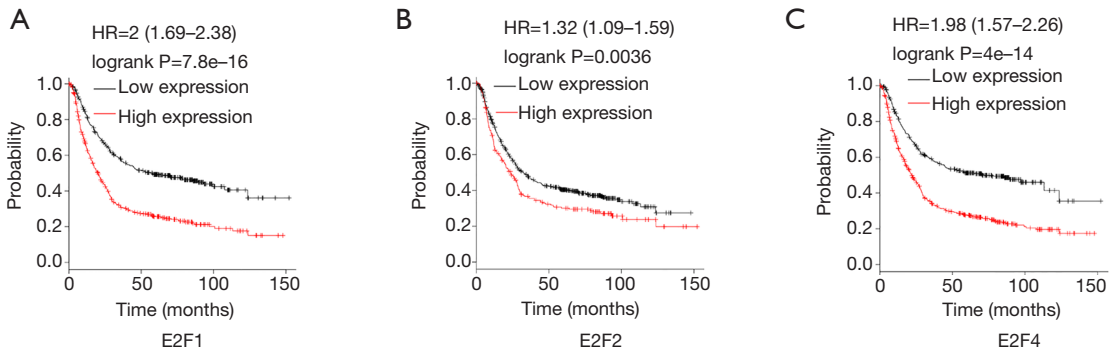

Figure 6 High levels of E2F1/2/4 factor mRNA expression improved the survival probability of patients with GC. (A,B,C) The effect of high levels of E2F1/2/4 factor mRNA expression on the survival probability of gastric cancer patients using the Kaplan-Meier plotter. GC, gastric cancer.

the prognosis of patients with GC.

Discussion

It has been reported that E2Fs are dysregulated in various types of cancers (27-29). Recently, several reports have shown that E2Fs are involved in the tumorigenesis and prognosis of several cancers. However, few bioinformatics analyses of E2Fs have been performed. In this study, we showed the prognostic values of E2F1/2/4 in GC.

E2F1 is the most researched and investigated among all E2F members in the context of human cancers (30). Several studies indicate that E2F1 expression was significantly associated with a poor prognosis in several malignancies, including pancreatic, esophageal, and non-small cell lung cancer. Also, E2F1 has been reported to be a tumour suppressor in GC (31). However, in the present study, E2F1 was associated with the cell cycle and played a role in immune infiltration in GC.

E2F2 has been shown to play a critical role in several cellular processes, including differentiation, cell cycle, proliferation, and cancer development (32-34). As shown in a recent report, E2F2 was highly expressed in lung cancer tissues compared with normal tissues (35). Polymorphisms in the E2F2 promoter are associated with an increased risk of squamous cell carcinoma in the oropharynx, and various cancers were affected by E2F2 expression (36). In our study, low E2F1/24 expression was correlated with a longer OS in GC patients.

In patients with breast cancer, the E2F4 target genes exhibited high expression, which led to shorter survival and the development of more severe cancer (37). The methylation of E2F4 might also be of prognostic value in breast cancer through influencing its expression (38). Moreover, lung cancer patients with high expression of E2F4 showed a poor OS. In this study, high E2F4 expression was significantly correlated with immune infiltration, including macrophages, neutrophils, and DCs, which might have led to the poor OS of GC patients.

In this study, we analyzed and determined the expression and prognostic value of E2F1/2/4 in GC patients. We further investigated the complexity and heterogeneity of the molecular properties associated with GC. Additionally, increased E2F1/2/4 expression was found to play a special role in the cell cycle through interacting with CDK2/4/6. Our findings also suggested that the level of E2F1/2/4 expression was negatively correlated with infiltrating levels of B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and DCs in GC. Thus, high E2F1/2/4 expression may also serve as a biomarker that can be used to identify high-risk subgroups of patients with GC.

Conclusions

We showed that E2F1/2 was highly expressed in the serum of GC patients. As predicted by bioinformatics, E2F1/2/4 was correlated with patient prognosis and immune cell infiltration and can be used as a prognostic biomarker and indicator of immune infiltration in GC.

Acknowledgments

Funding: This study was supported by the Zhejiang Province Public Welfare Technology Application Research

Project (No. 2020KY058) and Zhejiang Province Public Welfare Technology Application Research Project (No. 2021KY082).

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://dx.doi.org/10.21037/tcr-21-45

Data Sharing Statement: Available at https://dx.doi.org/10.21037/tcr-21-45

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/tcr-21-45). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Zhejiang Cancer Hospital (No. IRB -2020-407). Individual consent for this retrospective analysis was waived

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Cite this article as: Chen Y, Gong W, Dai W, Jiang H, Xu X. E2F1/2/4 mRNA is associated with immune infiltration and are potential biomarkers for the prognosis of human gastric carcinoma. Transl Cancer Res 2021;10(6):2801-2811. doi: 10.21037/tcr-21-45

