Original Article

Low CPEB1 levels may predict the benefit of 5-fluorouracil treatment in patients with colon or stomach adenocarcinoma

Jing-Zhu Cao[1#], Dan-Dan Niu[2#], Zhi-Ping Huang[3#], Yong-Gang Hong[4#], Zhen-Guang Wang[5], Le Yang[5], Bo-Lun Zhao[6], Guang-Lei Qiao[7], Liu Ouyang[8]

[1]Department of Endocrinology and Metabolism, Changhai Hospital, Naval Medical University, Shanghai, China; [2]Department of Anesthesiology, Huashan Hospital, Fudan University, Shanghai, China; [3]Department of Hepatobiliary Surgery, General Hospital of Southern Theatre Command, Guangzhou, China; [4]Department of Colorectal Surgery, Changhai Hospital, Naval Medical University, Shanghai, China; [5]The Third Department of Hepatic Surgery, Eastern Hepatobiliary Surgery Hospital, Naval Medical University, Shanghai, China; [6]School of Nursing, Dalian University, Dalian, China; [7]Department of Oncology, Tongren Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; [8]Department of the HBP Surgery, Changhai Hospital, Naval Medical University, Shanghai, China

Contributions: (I) Conception and design: GL Qiao, BL Zhao, L Ouyang; (II) Administrative support: GL Qiao; (III) Provision of study materials or patients: JZ Cao; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Liu Ouyang. Department of the HBP Surgery, Changhai Hospital, Naval Medical University, Shanghai 200433, China. Email: aqqwbjsqtb@163.com; Guang-Lei Qiao. Department of Oncology, Tongren Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200336, China. Email: qiaoguanglei26@126.com; Bo-Lun Zhao, School of Nursing, Dalian University, Dalian 116000, China. Email: zhaobolun@dlu.edu.cn.

Background: For patients with colon or stomach adenocarcinoma, 5-fluorouracil (5-FU) is an essential component of systemic chemotherapy in the palliative and adjuvant settings. The post-transcriptional regulatory factor cytoplasmic polyadenylation element-binding protein 1 (CPEB1) has been reported to be linked to tumor metastasis. This study aimed to investigate the relationship between CPEB1 expression and 5-FU treatment response in patients with colon and stomach adenocarcinomas.

Methods: The expression of *CPEB1* in stomach adenocarcinoma and colorectal cancer (CRC) tissues and in cell lines was determined by quantitative real-time PCR (qRT-PCR) and immunohistochemistry analyses. Transwell assays were employed to analyze the effects of *CPEB1* on the migration and invasion abilities of gastric cancer (GC) and CRC cells.

Results: The expression levels of *CPEB1* were increased in colon and stomach adenocarcinoma and were negatively correlated with malignancy and poor patient survival. Data suggested that patients with CRC or GC who had strong CPEB1 expression responded poorly to 5-FU treatment. Furthermore, knockdown of CPEB1 inhibited the migration and invasion of CRC and GC cells via a mechanism involving decreased expression of matrix metalloprotein (*MMP*)2, 7, and 9. Finally, our methylated RNA immunoprecipitation PCR (meRIP qPCR) data suggested that the increased CPEB1 expression in colon and stomach adenocarcinomas might be mediated by FTO (FTO alpha-ketoglutarate dependent dioxygenase)-dependent m^6A demethylation of CPEB1 mRNA.

Conclusions: Our results indicate that the level of CPEB1 expression may be valuable for predicting the benefit of 5-FU treatment for patients with colon and stomach adenocarcinomas. We therefore propose that low CPEB1 expression may represent a novel biomarker for personalized 5-FU therapy.

Keywords: Cytoplasmic polyadenylation element-binding protein 1 (*CPEB1*); colon adenocarcinoma; stomach adenocarcinoma; biomarker; 5-fluorouracil treatment (5-FU treatment)

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Introduction

Colon adenocarcinoma and stomach adenocarcinoma are two of the most common cancers globally. Since its discovery in 1957, 5-fluorouracil (5-FU) has been used as a first-line treatment for colorectal cancer (CRC) (1-4). Nevertheless, the efficacy of 5-FU-based chemotherapy for CRC is disappointing, as most patients acquire drug resistance during treatment, resulting in a median survival time of only about 20 months (5,6). Furthermore, for patients with gastric cancer (GC), the benefits of palliative chemotherapy and supportive treatment are limited, with a response rate ranging from 25% to 50% and the median survival being 6 to 12 months (7). Therefore, chemoresistance is the main obstacle to improving the therapeutic effect of 5-FU in patients with CRC and GC.

Cytoplasmic polyadenylation element-binding protein 1 (CPEB1) binds to cytoplasmic polyadenylation elements (CPEs) of the specific mRNA 3'-untranslated region (UTR) by anchoring the atypical poly(A) polymerases Gld2 and Gld4 and the deadenylase poly(A)-specific ribonuclease (PARN) (8,9). This binding regulates the addition or removal of poly(A) tails, thereby promoting or inhibiting translation, and is particularly important for regulating mRNAs involved in cell cycle G2/M progression (10,11). Decreased *CPEB1* levels are implicated in cell invasion and angiogenesis in several cancers (12). Knockout of *CPEB1* results in metastasis-related mRNAs having shorter or longer poly(A) tails. In breast cancer, *CPEB1* levels decrease when cells metastasize (13). There is also strong evidence that *CPEB1* regulates the differentiation of glioma stem cells and inhibits the proliferation of glioblastoma cells (14,15). Moreover, recent study has shown that *CPEB1* mediates stem cell chemoresistance in hepatocellular carcinoma (16). However, the relationship between *CPEB1* expression and 5-FU treatment response in colon and gastric adenocarcinoma have not been examined.

In the present study, we analyzed the relationships between 5-FU targets and the prognosis of patients treated with this drug. Our results indicate that CPEB1 may be a new predictor of the efficacy of 5-FU in patients with colon or gastric adenocarcinoma. We present the following article in accordance with the MDAR reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-561/rc).

Methods

Patients and treatment

Nine patients with colorectal adenocarcinoma and six patients with gastric adenocarcinoma who were treated at Changhai Hospital were enrolled in this study. Tumor and adjacent normal tissues from these patients were subjected to quantitative real-time PCR (qRT-PCR) and m^6A methylated RNA immunoprecipitation quantitative PCR (meRIP qPCR). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Changhai Hospital (No. 16ZR1400800). Written informed consent was obtained from each participant.

Immunohistochemistry

Immunohistochemistry assays were performed as previously described (17). The staining intensity for each marker was scored visually using the following four-point scale: 0= no staining, 1= weak staining, 2= moderate staining, and 3= strong staining. Based on this score, the H-score was calculated for the percentage of positive cells, on a scale from 0 (no staining) to 300 (diffuse, strong staining).

RT-PCR

Total RNA isolation and RT-PCR were performed as previously described (17). All expression values were normalized to β-actin expression.

m^6A meRIP qPCR

MeRIP analysis was performed according to the manufacturer's instructions (Magna meRIP m^6A). The

beads were then washed and eluted with 5'-sodium monophosphate (m^6A) to retrieve the bound RNA. The eluted RNA was purified using miRNeasy miniature kits (QIAGEN, German) and finally, qPCR was performed.

Cell lines

The CRC cell lines HCT116 and LOVO and the GC cell lines MGC-803 and GBC-803 were purchased from the American Type Culture Collection Center (ATCC; Manassas, VA, USA) and cultured at 37 ℃ in Eagle's Minimum Essential Medium, as specified by the ATCC (catalog No. 30-2003; ATCC) with 10% fetal bovine serum (FBS; Sangon Biotech Co., Ltd., Shanghai, China).

Cell transfection

Synthesis of si-FTO, si-CPEB1, and si-Con was performed by Shanghai Gene Pharmaceutical Co., Ltd. Cells were transfected with the siRNAs (50 nM) at 37 ℃ using Lipofectamine™ 2000 (Invitrogen, USA; Thermo Fisher Scientific, Inc., USA). At 48 hours after transfection, cells were collected for subsequent analyses. siRNA transfections were si-Con: 5'-UGAGGACCUGGGU-3'; si-CPEB1: 5'-AUCUGAUCCAGAGCUGAAGCC-3'; si-FTO 5'-UGUGAUCCAGAGCUGAAGCC-3'.

Cell migration and invasion assays

The migration and invasion abilities of GC and CRC cells were examined as previously described (16). The cells were inoculated into 500 μL FBS-free DMEM in the upper lumen (2×10^4/well) and DMEM with 10% FBS in the lower lumen. The cells were cultured at 37 ℃ and 5% CO_2 for 1 day. For the Matrigel intrusion test, the Transwell chamber was coated with Matrigel (BD Biosciences, San Jose, CA, USA). A total of 5×10^4 transfected cells were inoculated into the upper lumen of Transwell in 200 μL medium without FBS, while 500 μL medium supplemented with 20% FBS was placed into the lower lumen. The cells were cultured at 37 ℃ for 24 h for migration test and 48 h for invasion test. In both tests, cells were immobilized with 100% methanol for 5 min (Haimen Beotimi Biotechnology Institute, China) and stained with 0.5% crystal violet for 5 min (Beotimi Biotechnology Institute, China). The remaining cells on the upper surface of the membrane are then carefully removed with a cotton swab. The migrating and invading cells were then counted using an inverted microscope (magnification ×200) in five randomly selected areas. Each experiment was repeated three times.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). The baseline clinicopathological features of the patients were analyzed using the chi-squared test. Survival was estimated using the Kaplan-Meier method and compared by logarithmic rank testing. P value <0.05 was considered statistically significant.

Results

CPEB1 expression is increased in colon and stomach adenocarcinoma

Analysis of data from the GEPIA2 database (http://gepia2.cancer-pku.cn/#index) revealed that patients whose tumors expressed high levels of *CPEB1* had shorter overall and disease-free survival (*Figure 1A*,*1B*). Results of IHC examination indicated that CPEB1 was also increased at the protein level in colon and stomach adenocarcinoma tissues relative to the adjacent normal tissues. Moreover, a high *CPEB1* protein level was associated with poor 5-FU response in the 9 patients with colon adenocarcinoma and 6 with stomach adenocarcinoma (*Figure 2A*,*2B*). This observation evidences a clear association between the level of CPEB1 and 5-FU treatment benefit in patients with relapsed colon or stomach adenocarcinoma.

Effects of CPEB1 on metastatic potential and invasive behavior of GC and CRC cells in vitro

To functionally characterize the effects of *CPEB1* in GC and CRC, the CRC cell lines HCT116 and LOVO and the GC cell lines MGC-803 and GBC-803 were selected for a loss-of-function study. To this end, GC and CRC cells were stably downregulated for CPEB1, and the knockdown of CPEB1 expression was confirmed by qRT-PCR (Figure S1A,S1B). Transwell assays revealed that knockdown of CPEB1 decreased the migration and invasion abilities of GC and CRC cells (*Figure 3A-3D*). These data indicate that CPEB1 may promote GC and CRC cell metastasis *in vitro*.

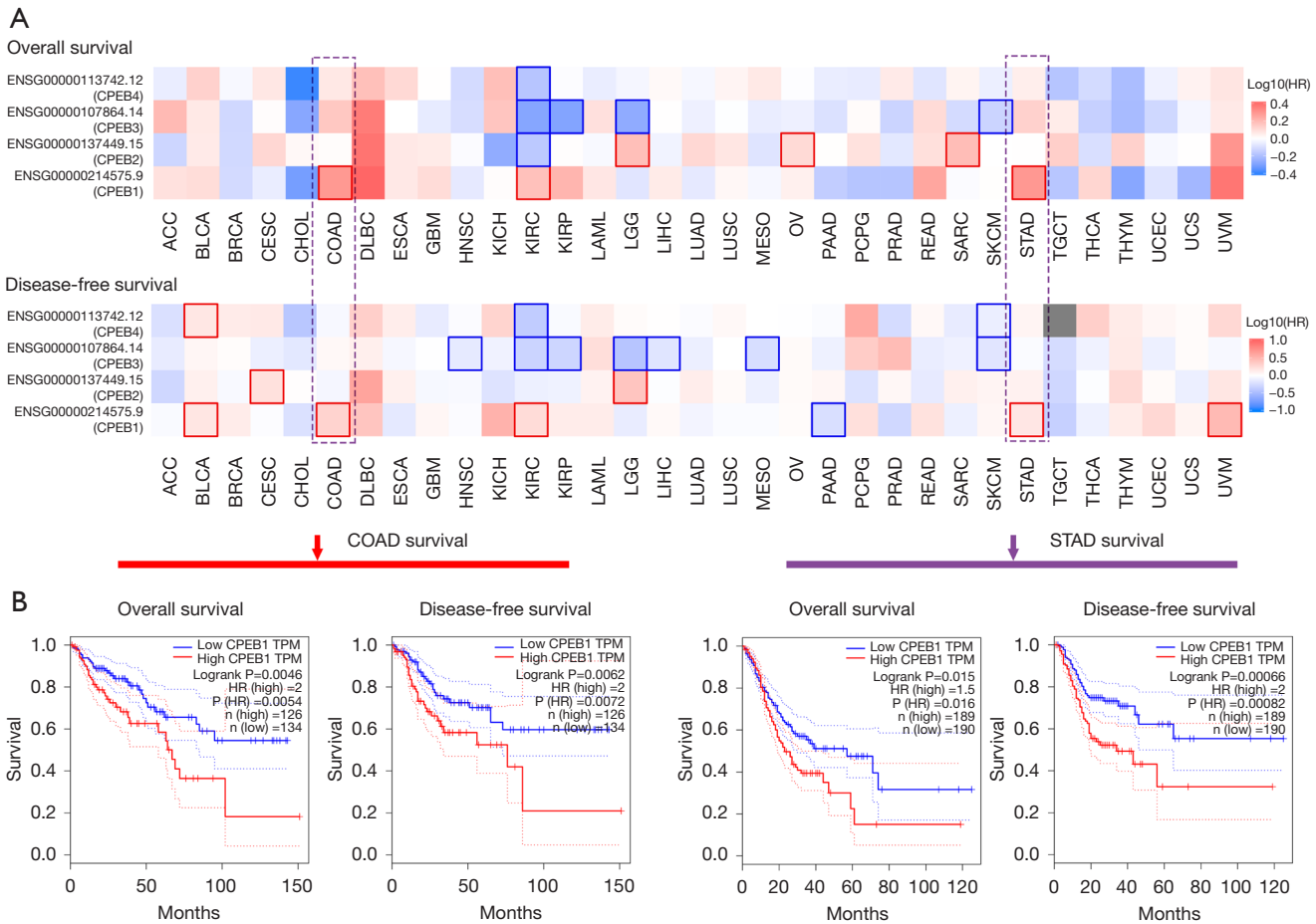


Figure 1 CPEB1 expression is increased in colon and stomach adenocarcinoma. (A,B) Kaplan-Meier estimates of overall survival and disease-free survival based on data extracted from the GEPIA2 database and stratified into subgroups according to CPEB1 expression. CPEB1, cytoplasmic polyadenylation element-binding protein 1; COAD, colon adenocarcinoma; STAD, stomach adenocarcinoma; TPM, transcripts per million; HR, hazard ratio.

CPEB1 regulates the expression of matrix metalloprotein (MMP)2, 7, and 9 in GC and CRC cells

Tumor invasion is orchestrated by an increase in the proteolytic activity of MMPs, which degrades the neighboring stroma and facilitates the spread of tumor cells. Using the GEPIA2 database (http://gepia2.cancer-pku.cn/#index) we found that high CPEB1 expression was positively associated with the expression of *MMP2*, 7, and *9* in GC and CRC tissues (*Figure 4A,4B*). A marked decrease in *MMP2*, 7, and *9* protein expression was also observed in GC and CRC cell lines after CPEB1 knockdown (*Figure 4C,4D*). These observations suggest that, at least in part, tumor metastasis is positively influenced by CPEB1 via its regulation of *MMP2*, 7, and *9* expressions in GC and CRC cells.

FTO-induced m^{6}A modification is responsible for increasing CPEB1 expression in colon and stomach adenocarcinoma

Again, using the GEPIA2 database, we analyzed the correlations between the expression of CPEB1 and that of methylases (*METTL3*, *METTL14*, *WTAP*, and *KIAA1429*) and demethylases (*ALKBH5* and *FTO*). Results showed that the m^{6}A demethylase FTO was positively associated

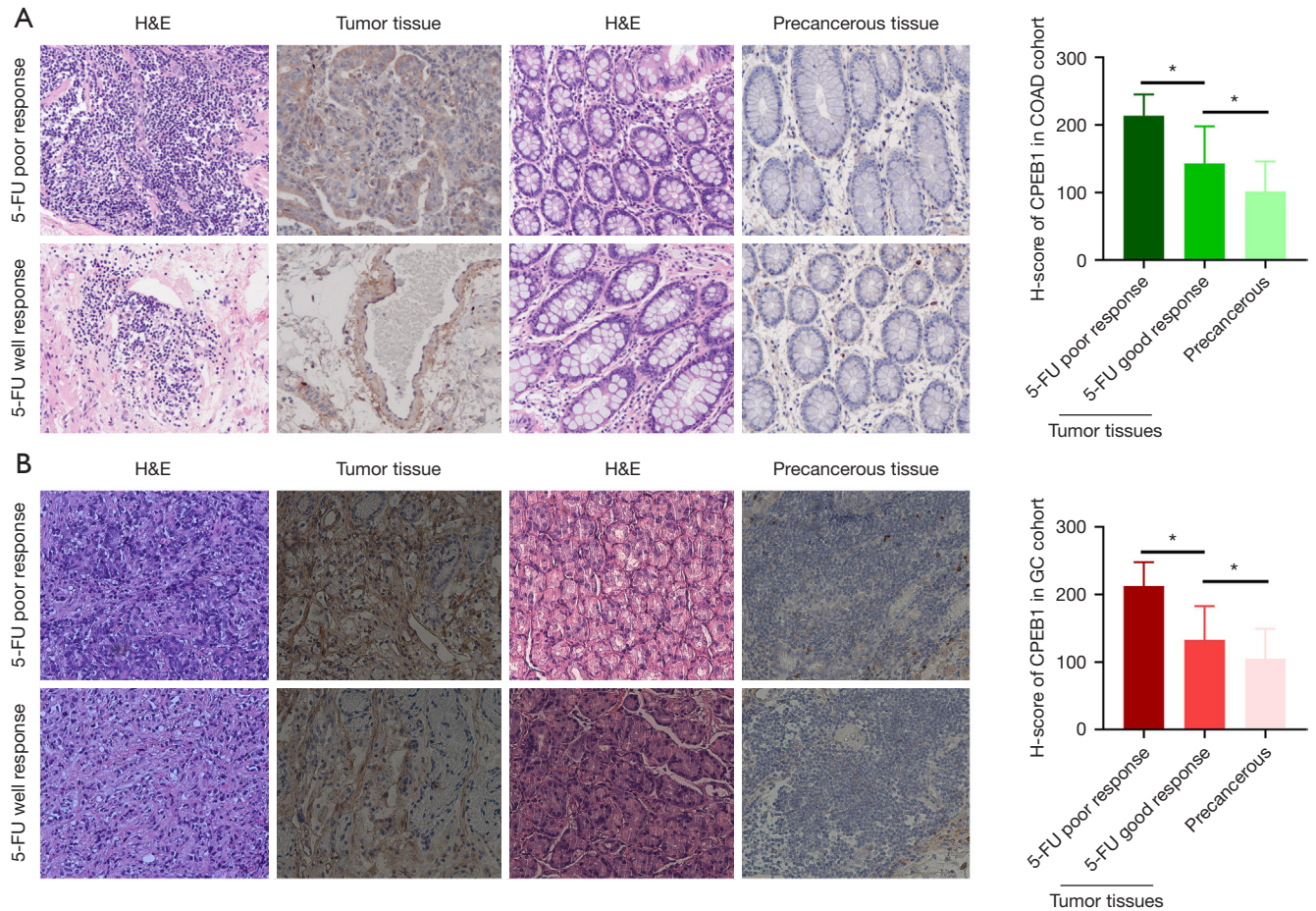


Figure 2 Low CPEB1 levels confer 5-FU sensitivity in colon adenocarcinoma and stomach adenocarcinoma. Immunohistochemical staining of CPEB1 in nine paired primary colon adenocarcinoma tissues (A) and six paired stomach adenocarcinoma tissues (B). Magnification ×200. Data are shown as mean ± SD. *P<0.05. 5-FU, 5-fluorouracil; H&E, hematoxylin and eosin; CPEB1, cytoplasmic polyadenylation element-binding protein 1; COAD, colon adenocarcinoma; GC, gastric cancer; SD, standard deviation.

with *CPEB1* in both colon and stomach adenocarcinoma (*Figure 5A*). Knockdown of *FTO* significantly decreased the expression of CPEB1 (*Figure 5B*), which suggested that m^{6}A modification could be involved in the increase levels of CPEB1 in colon and stomach adenocarcinoma cells. In subsequent study, a RIP assay validated m^{6}A modification of CPEB1 mRNA in colon and stomach adenocarcinoma cells and also confirmed the role played by FTO in this modification (*Figure 5C*). As shown in *Figure 5D*, the expression levels of FTO and CPEB1 were increased in tumor tissues compared to their adjacent normal tissues (*Figure 5D,5E*). Moreover, meRIP qPCR analysis of 9 paired colon and 6 paired stomach adenocarcinoma samples revealed that the m^{6}A levels of CPEB1 were lower in tumor tissues than in the adjacent normal tissues (*Figure 5F*). Consistent with this result, a positive correlation between FTO expression and CPEB1 levels was observed (*Figure 5G*). These results suggest that FTO-induced m^{6}A modification is responsible for the increased expression levels of CPEB1 in colon and stomach adenocarcinoma.

Discussion

The post-transcriptional regulatory factor CPEB1 is involved in cytoplasmic polyadenylation, which may influence tumorigenesis in various malignant tumors (18). However,

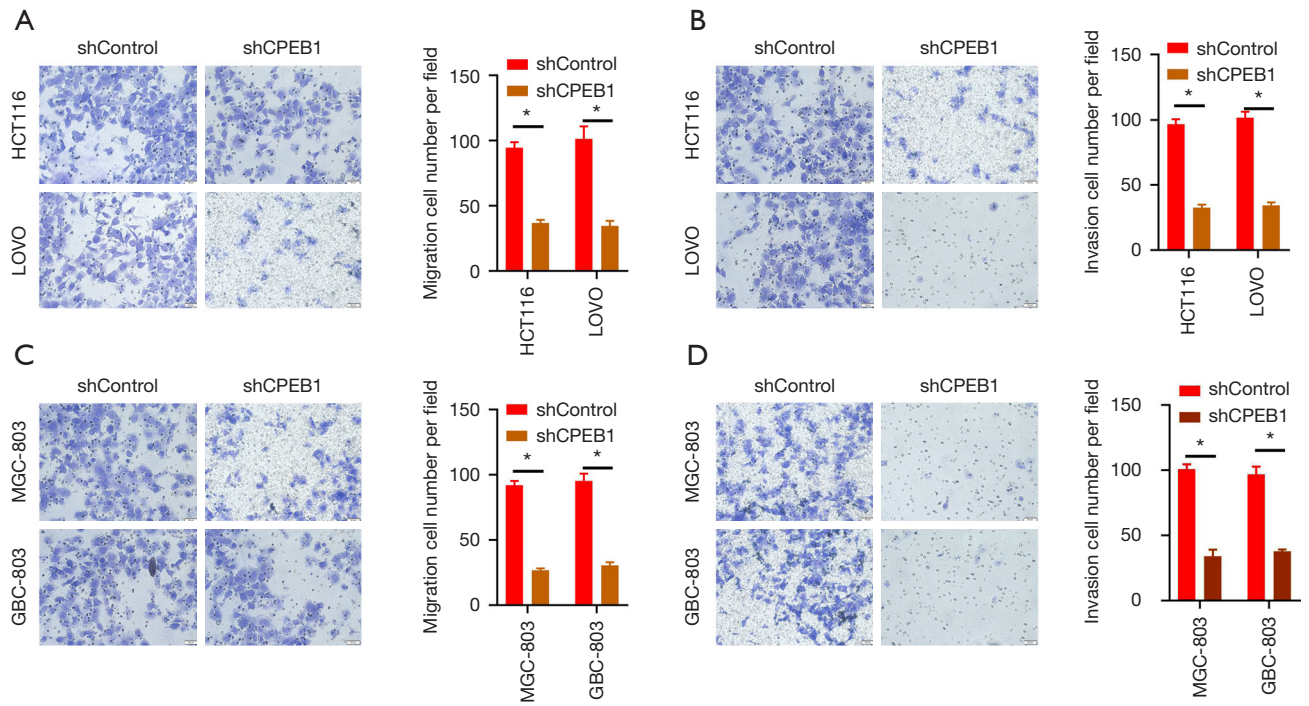


Figure 3 Effects of CPEB1 on the metastasis and invasion of GC and CRC cells *in vitro*. Results of Transwell migration (A) and Matrigel invasion (B) assays of the CRC cell lines HCT116 and LOVO. Results of Transwell migration (C) and Matrigel invasion (D) assays of the GC cell lines MGC-803 and GBC-803. 0.1% crystal violet was used to stain the cells. Cell counts from three randomized fields at a magnification of ×100. *P<0.05. CPEB1, cytoplasmic polyadenylation element-binding protein 1; GC, gastric cancer; CRC, colorectal cancer.

the expression and role of CPEB1 in colon adenocarcinoma and stomach adenocarcinoma have remained largely unknown. Based on the results of the present study, we propose that CPEB1 is markedly upregulated in colon and stomach adenocarcinoma tissues via FTO-dependent m^{6}A RNA demethylation machinery. We found that increased CPEB1 expression was correlated with poor survival in patients with colon and stomach adenocarcinoma. Moreover, patients with high CPEB1 expression exhibited a poor response to 5-FU treatment.

Although patient life expectancy can be significantly extended by 5-FU-based chemotherapy, drug resistance is a major barrier to the success of this treatment (19). Due to complex and heterogeneous biological processes, many of the mechanisms of chemoresistance remain elusive (20). Therefore, there is a need to identify novel biomarkers for patient selection to improve the efficacy of 5-FU. Our clinical investigation revealed that patients with colon or stomach adenocarcinoma who had low CPEB1 levels benefited from 5-FU therapy, whereas those with high CPEB1 levels did not. Collectively, these findings suggest that CPEB1 may serve as a predictor of 5-FU benefit in the personalized therapy of colon and stomach adenocarcinoma, which warrants further investigation.

Emerging studies have demonstrated that m^{6}A modification of mRNAs plays a critical role in RNA fate, including mRNA stability, splicing, transport, localization, and translation (21-26). Previous studies indicated that N6-methyladenosine was associated with 5-FU resistance in CRC (27,28). Demethylation of m^{6}A has been reported to be modulated by FTO and ALKBH5 (25-33). Based on the results of the present study, we propose that CPEB1 expression in colon and stomach adenocarcinoma is regulated by RNA methylation, as assessed using the GEPIA2 database and our observations that interference with FTO resulted in an increase of m^{6}A leading to a decrease of CPEB1. Analysis of clinical samples of colon and stomach adenocarcinoma revealed that the levels of

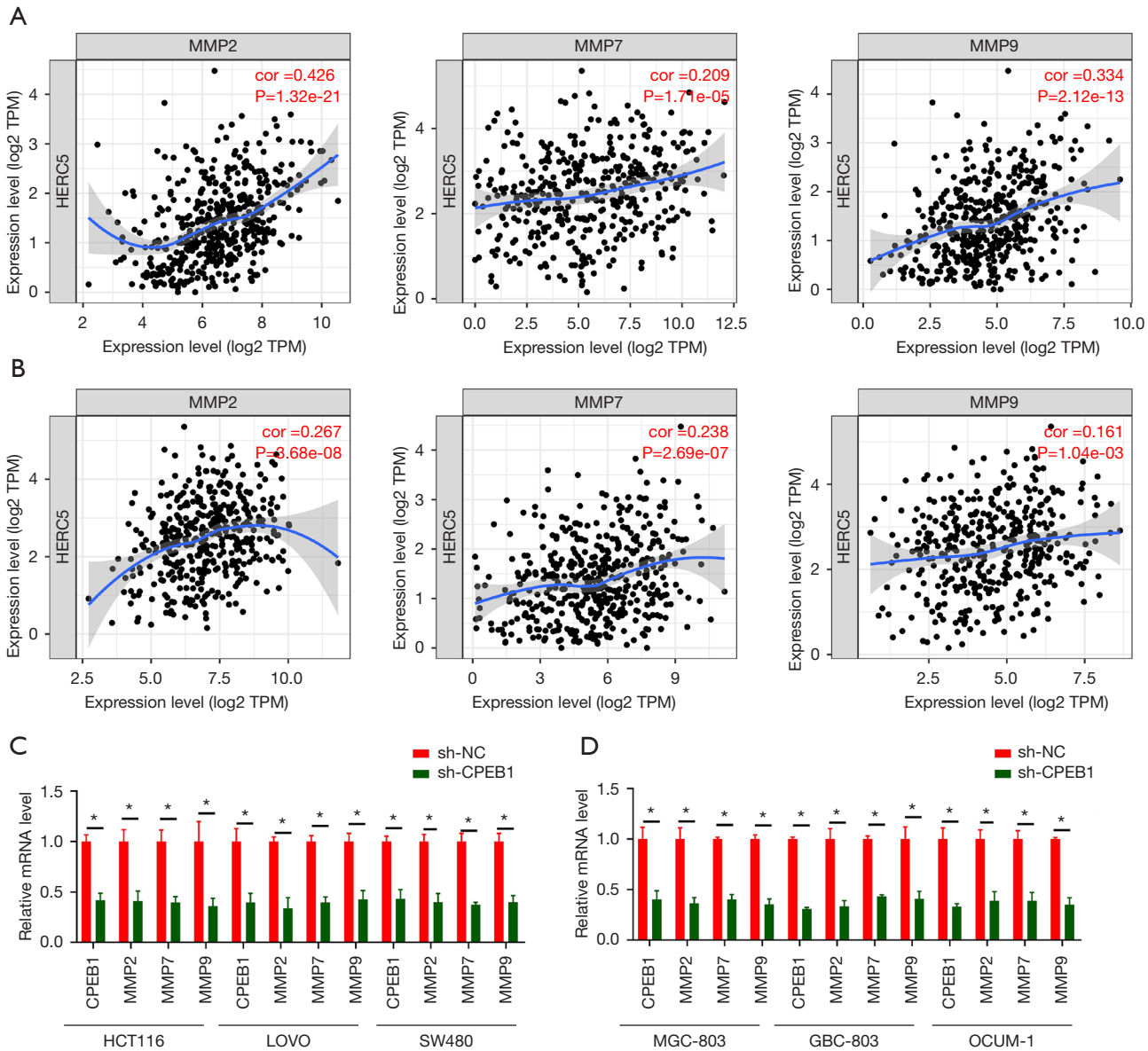
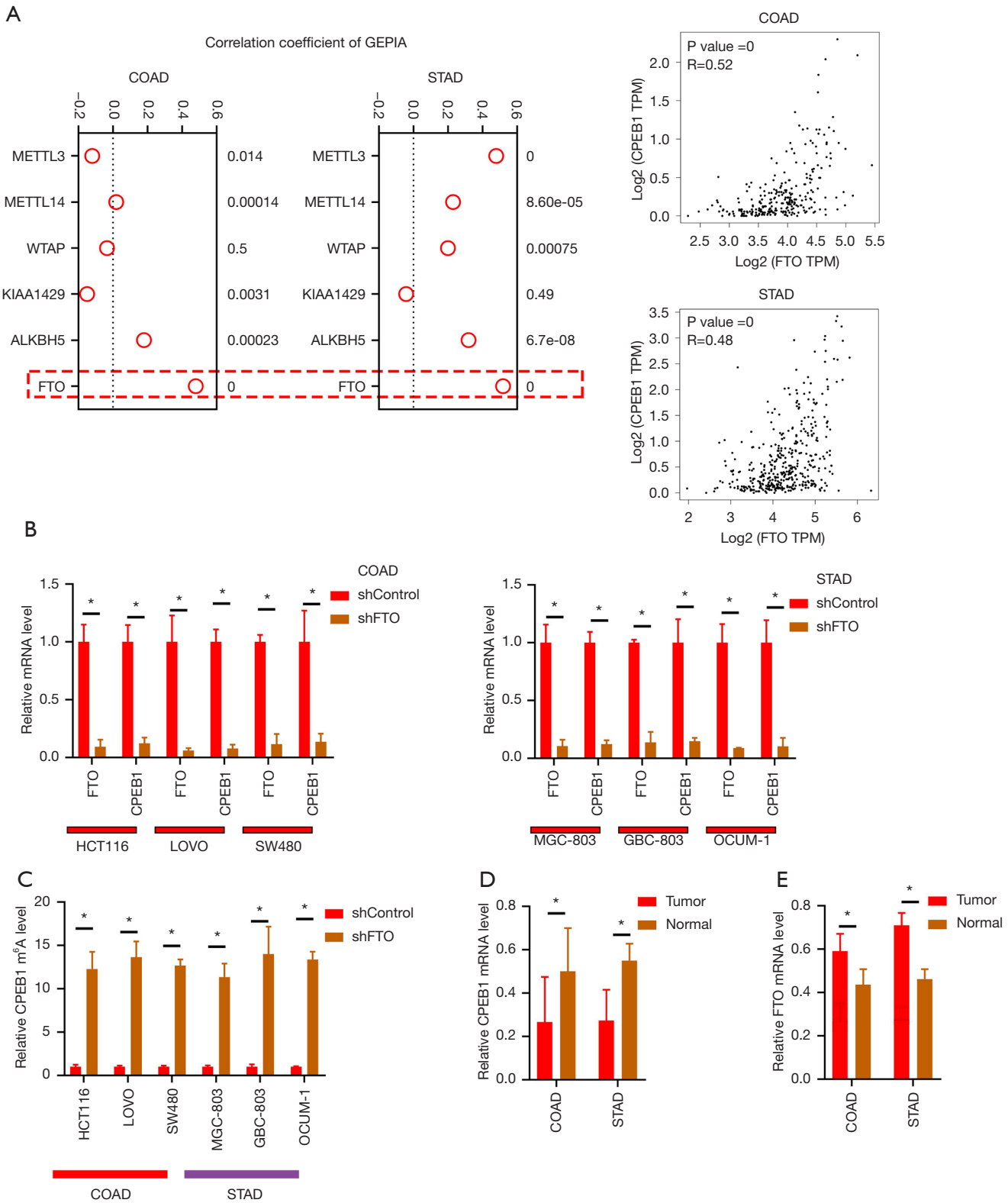


Figure 4 CPEB1 regulates the expression of MMP2, 7, and 9 in GC and CRC cells. (A,B) Co-expression of MMP2, 7, 9 and CPEB1 in GC and CRC tissues in the GEPIA2 database. (C,D) Expression of MMP2, 7, 9 in GC and CRC cells after CPEB1 knockdown. *P<0.05. TPM, transcripts per million; MMP, matrix metalloprotein; CPEB1, cytoplasmic polyadenylation element-binding protein 1; NC, normal control; GC, gastric cancer; CRC, colorectal cancer.

CPEB1 and FTO mRNA were positively correlated, while the m^{6}A CPEB1 level was negatively correlated with the levels of CPEB1 and FTO mRNA. However, the molecular mechanisms underlying the decrease of CPEB1 induced by m^{6}A modification in colon and stomach adenocarcinoma cells remain obscure. To the best of our knowledge, this is the first study to report the involvement of m^{6}A modification in CPEB1 dysregulation in GC and CRC.

In conclusion, we have revealed the value of CPEB1 in predicting the benefit of 5-FU treatment in colon or stomach adenocarcinoma. On this basis, we propose that low CPEB1 expression could serve as a novel biomarker for the use of 5-FU in the personalized therapy of patients with colon or stomach adenocarcinoma.



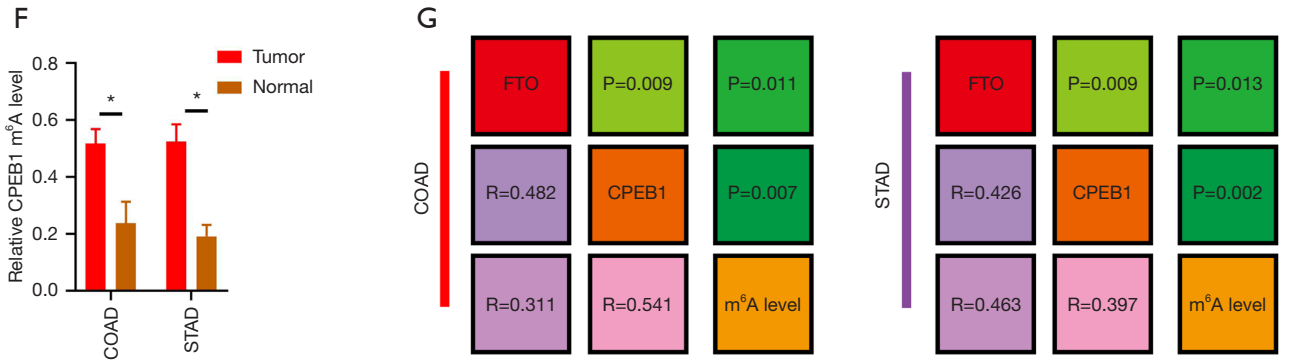


Figure 5 FTO-induced m^{6}A modification is responsible for increasing CPEB1 in colon and stomach adenocarcinoma. (A) Correlations of *CPEB1* expression with the expression of methylases (*METTL3*, *METTL14*, *WTAP*, and *KIAA1429*) and demethylases (*ALKBH5* and *FTO*) in primary colon adenocarcinoma and stomach adenocarcinoma, using data from the GEPIA database. (B) FTO and CPEB1 mRNA expression in colon adenocarcinoma and stomach adenocarcinoma cells transfected with sh-control or sh-FTO. (C) The m^{6}A levels of CPEB1 in colon adenocarcinoma and stomach adenocarcinoma cells transfected with sh-control or sh-FTO. (D,E) FTO and CPEB1 mRNA expression in colon adenocarcinoma and stomach adenocarcinoma (tumor and adjacent normal tissues) by RT-PCR. (F) The m^{6}A levels of CPEB1 in colon adenocarcinoma and stomach adenocarcinoma (tumor and adjacent normal tissues) by meRIP qPCR. (G) Correlations of mRNA CPEB1, FTO, and m^{6}A CPEB1 expression in 9 paired primary colon adenocarcinoma and 6 paired stomach adenocarcinoma tissues based on data from the GEPIA database. *P<0.05. COAD, chronic obstructive pulmonary disease; STAD, stomach adenocarcinoma; TPM, transcripts per million; CPEB1, cytoplasmic polyadenylation element-binding protein 1; RT-PCR, real-time PCR; meRIP qPCR, methylated RNA immunoprecipitation quantitative PCR.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-561/rc

Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-561/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-561/coif). 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 Changhai Hospital (No. 16ZR1400800). Written informed consent was obtained from each participant.

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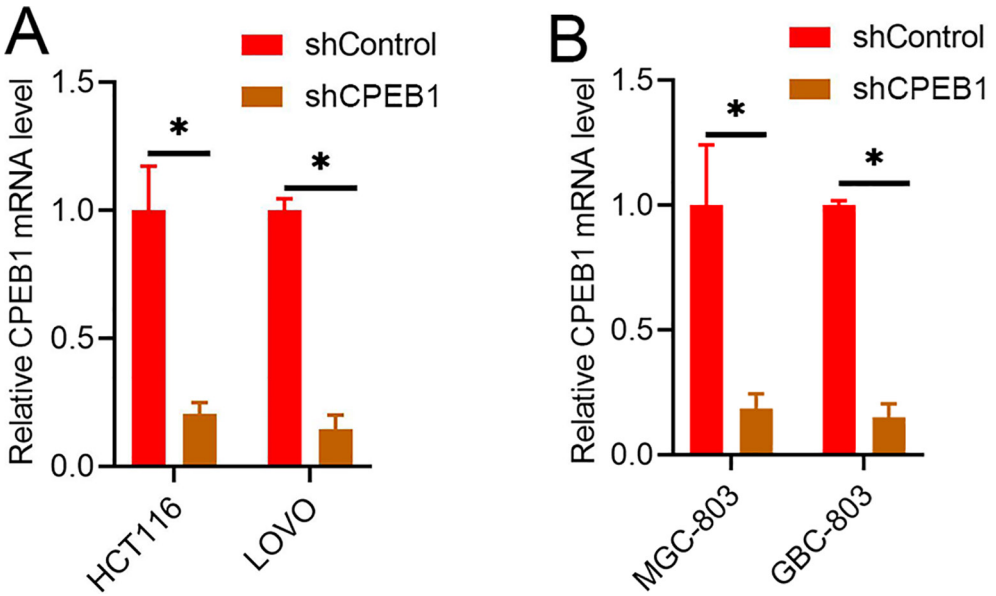

Figure S1 Effects of *CPEB1* on the metastatic potential and invasive behavior of GC and CRC cells *in vitro*. (A,B) Expression of *CPEB1* mRNA in colon adenocarcinoma and stomach adenocarcinoma cells transfected with sh-control or sh-CPEB1. *P<0.05. CPEB1, cytoplasmic polyadenylation element-binding protein 1; GC, gastric cancer; CRC, colorectal cancer.

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