Original Article
MCM4 upregulation predicts poor prognosis in gastric cancer

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Abstract: Mini-chromosome maintenance protein 4 (MCM4) is essential for the initiation of DNA replication. However, the expression and prognostic values of MCM4 in gastric cancer (GC) are still unclear. In our study, MCM4 expression in GC was determined by using the ONCOMINE database. The prognostic values of MCM4 in GC patients was explore by using Kaplan-Meier plotter database. A network of MCM4 interactions with other molecules was predicated in STRING and GEPIA databases. Moreover, we explored the roles of MCM4 on GC progression. In the present study, we showed that MCM4 expression was significantly upregulated in GC tissues both in mRNA and protein levels. KM Plotter showed that high MCM4 expression was associated with poor overall survival (OS) in GC patients. STRING and GEPIA databases showed that 10 mRNAs might have an interaction with MCM4 in GC. In addition, our data showed that MCM4 expression was upregulated in GC tissues and high MCM4 expression was associated with advanced TNM stage and lymph node metastasis. In vitro function assays showed that decreased MCM4 expression significantly suppressed GC cells proliferation and arrested gastric cancer cells cycle in G0/G1 phase. Therefore, these data suggested that MCM4 could act an oncogene in GC progression, which might serve as a novel molecular target in GC therapy.

Keywords: MCM4, gastric cancer, prognosis, KM plotter

Introduction

Gastric cancer (GC) is a common malignancy and the second leading cause of cancer-related deaths, worldwide [1, 2]. Although patients during the early stages of GC can be cured by surgery, most GC patients are diagnosed at advanced stages and present with extensive invasion, lymphatic metastasis, and other organ metastases [3, 4]. Thus, clinical outcomes of GC patients have remained unsatisfactory [5]. Therefore, it is important to identify new additional prognostic markers, aiming to improve patient prognosis.

The mini-chromosomal maintenance (MCM) protein family consists of 6 related proteins (MCM2-7). These are involved in the elongation of DNA replication and other chromosome transactions, including damage response, transcription, and chromatin structure [6-8]. Aberrant expression of MCM proteins has been reported to be a promising prognostic marker in many malignancies [9, 10]. For example, Gonzalez et al. showed that MCM2 was upregulated and associated with advanced clinical features, overall survival, and disease-free interval of breast cancer patients [11]. Toyokawa et al. showed that MCM7 was increased in non-small cell lung cancer and could act as a potential therapeutic target and a novel prognostic marker [12]. Schrader et al. showed that high MCM6 expression indicated early G1-phase arrest, as a new prognostic marker in mantle cell lymphoma [13]. Lau et al. showed that MCM 2, 3, and 7 were overexpressed in medulloblastoma and were involved in the regulation of cell migration and invasion [14]. However, the expression and prognostic value of MCM4 are unclear in GC patients.
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Materials and methods

ONCOMINE analysis

First, mRNA levels of MCM4 in different cancers were analyzed using ONCOMINE datasets (www.oncomine.org) [15]. This study compared clinical specimens of cancer versus normal control datasets, using Student’s t-test to generate a P value. The P value was set up at 0.0001 and fold change was defined as 2. The data type was restricted to mRNA.

Kaplan-Meier plotter survival analysis

KM Plotter online biomarker analysis tool (http://www.kmplot.com/) was used to produce Kaplan-Meier plots for overall survival (OS), based on the signal intensity of Affymetrix ID 222037_at (MCM4) [16]. The dataset was divided into high and low expression groups using the auto-select best cutoff. Hazard ratios (HR) with 95% confidence intervals and log rank P values were calculated and displayed on the webpage.

Patient and sample collection

A total of 30 patients with GC were collected from Luoyang Center Hospital Affiliated to Zhengzhou University. None of the patients received chemotherapy and/or radiotherapy before surgery. Written informed consent was obtained from each participant or guardian. This case study was approved by the Ethics Committees of Luoyang Center Hospital Affiliated to Zhengzhou University.

Cell culture

Human gastric cancer cells MGC-803 and BGC-823 were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Thermo scientific, China) at 37°C containing 5% CO₂.

Small interfering RNA transfection

For knockdown of MCM4 in GC cell lines, small interfering RNA (siRNA) targeting MCM4 (si-MCM4) and a negative control siRNA (si-NC) were obtained from GenePharma (Shanghai, China). For transfection, cells were seeded in a 12-well plate with a density of 5×10⁶ cells/well. After 24 hours and 70%-80% confluence, cells were transfected with respective si-MCM4 and si-NC in a serum-free medium using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA), according to manufacturer instructions. After incubation for 6 hours at 37°C, the medium in each well was replaced with RPMI-1640 with 10% heat-inactivated fetal bovine serum for 48 hours.

RNA extraction and qRT-PCR analyses

Total RNA was extracted from tissues or cultured cells with TRizolReagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer instructions. RNA was reverse transcribed to cDNA from 1 μg of total RNA in a final volume of 20 μl using a Reverse Transcription Kit with gDNA Eraser (TaKaRa, Dalian, China). Expression levels of MCM4 were determined with quantitative RT-PCR (qRT-PCR) using SYBR Premix Ex Taq II Kit (TaKaRa, Dalian, China) in a LightCycler480 System. PCR was repeated in triplicate. GAPDH was used as an endogenous control in cell samples and snRNA U6 in tissue samples. Relative expression levels of MCM4 in GC tissues were calculated using 2⁻ΔΔCt method.

Cell proliferation assay

Cell proliferation was assessed using MTS assay (Promega, Madison, WI, USA). Cells (2,000/well) in each group were plated in 96-well plates. Next, 20 μl of the MTS reagents was added to each well containing 100 μl culture medium. The plate was incubated for 2 hours at 37°C in a humidified atmosphere containing 5% CO₂. The plate was read at 490 nm using a plate reader.

Cell cycle assay

At 48 h after transfection, GC cells were harvested and washed using ice-cold PBS solution. Subsequently the cells were fixed with 70% ethanol overnight at 4°C before being resuspended using PI/RNase A solution (5 μg/mL PI and 100 mg/mL RNase A) and incubated for
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Figure 1. MCM4 was increased in gastric cancer compared with normal gastric tissue. A. MCM4 mRNA expression (cancer vs. normal tissue) was analyzed with ONCOMINE database. The graphics demonstrate the numbers of datasets with statistically significant mRNA over-expression (red) or down-expression (blue) of the target gene. B, C. Comparison of MCM4 mRNA expression in Chen’s study. D, E. Comparison of MCM4 mRNA expression in Cho’s study. F, G. Comparison of MCM4 mRNA expression in D’Errico’s study. H. Comparison of MCM4 mRNA expression in Cui’s study. Abbreviation: gastric tissue (GT), gastric intestinal type adenocarcinoma (GITA), diffuse gastric adenocarcinoma (DGA), gastric mucosa (GM), gastric cancer (GC). The p value was set up at 0.0001 and fold change was defined as 2.
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15 min at room temperature in the dark. Then the flow cytometer was utilized to analyze cell cycle.

Statistical analysis

Statistical analyses were performed using SPSS version 18.0 (SPSS, Chicago, IL). Differences between groups were analyzed using Wilcoxon signed-rank test, Student’s t-test, or Chi-squared test, as indicated. P < 0.05 is considered statistically significant.

Results

MCM4 is distinctively upregulated in GC patients

MCM4 was identified in a number of human cancers, including gastric cancer (GC) (Figure 1). Oncomine analysis revealed that MCM4 mRNA was significantly overexpressed in GC. Chen's dataset [17] showed that MCM4 mRNA expression was higher in gastric intestinal type adenocarcinoma (GITA) and diffuse gastric adenocarcinoma (DGA) tissues than in gastric mucosa (GM) tissues (fold changes were 1.893 and 1.490, respectively) (Figure 1B and 1C). Cho's dataset [18] showed that MCM4 mRNA expression was higher in GITA and DGA tissues than in gastric tissues (GT) (fold changes were 1.812 and 1.854, respectively) (Figure 1D and 1E). In D’Errico’s dataset [19], MCM4 mRNA expression was decreased in GITA tissues and DGA tissues, compared with GM tissues (fold changes were 6.976 and 5.447; respectively) (Figure 1F and 1G). In Cui’s dataset [20], MCM4 mRNA was upregulated in GC tissues, compared with GT tissues (fold change was 2.116) (Figure 1H).

Next, this study explored MCM4 protein expression in clinical specimens from the human protein atlas (www.proteinatlas.org) database [22]. Results showed that MCM4 had a strong positive expression in GC tissues and a weak positive expression in gastric tissues (Figure 2A). Consistently, MCM4 mRNA expression was higher in the TCGA GC cancer RNAseq dataset (Figure 2B).
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A. GITA
B. DGA
C. MGA
D. HER2

E. moderately differentiated
F. poorly differentiated
G. well differentiated

I. Stage 1
J. Stage 2
K. Stage 3
L. Stage 4
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Figure 3. The prognostic value of MCM4 mRNA level in GC was from KM plotter. The desired Affymetrix ID is 222037_at (MCM4). (A-C) Survival curves are plotted for GITA (A), DGA (B), and MGA (C) patients. (D, E) Survival curves are plotted for HER2-negative (D) and HER2-positive (E) patients. (F-H) Survival curves are plotted for moderately differentiation (F), poor differentiation (G), and well differentiation (H) of patients. (I-L) Survival curves are plotted for clinical stages 1-4 (I-L) of patients.

Prognostic value of MCM4 in gastric cancer

The present study assessed the prognostic effects of MCM4 mRNA expression (Affymetrix ID is 222037_at) using www.kmplot.com. Survival curves were plotted in GC patients (n=876), GITA patients (n=320), DGA patients (n=241), Mixed Gastric Adenocarcinoma (MGA) patients (n=32), HER2-negative GC (n=532), HER2-positive GC (n=344), Well-differentiated GC (n=32), Moderately differentiated GC (n=67), Poorly differentiated GC (n=165), Stage 1 GC (n=13), Stage 2 GC (n=22), Stage 3 GC (n=85), and Stage 4 GC (n=43).

High MCM4 expression was associated with poor OS for all GC patients (Figure 2C). For further analysis, the patients were sub-grouped by types of cancer. High MCM4 was associated with poor OS in GITA, DGA, and MGA patients (Figure 3A-C). HER2 status in GC patients was reported as a potential target for individual therapy [23]. Thus, the survival curves were stratified by HER2 status to identify which patients may benefit from targeted detection. Present data showed that high MCM4 expression was significantly associated with poor OS in both HER2-negative and HER2-positive GC patients (Figure 3D, 3E). Next, it was shown that high transcriptional MCM4 expression was associated with poor OS in GC patients with moderately and poorly differentiation (Figure 3F, 3G), but not correlated with OS in GC patients with well-differentiation (Figure 3H). Furthermore, in clinical stages 1-4, high MCM4 mRNA expression was associated with poor OS in GC patients with different clinical stages (Figure 3I-L).

Prediction and annotation of MCM4 with other molecules’ network

Next, we explored other molecules which might associated with MCM4 by STRING database, and further confirmed it in GEPIA database. STRING database showed that 10 candidate mRNAs which might interacted with MCM4 in our study (Figure 4A). Subsequently, we explored the relationship between MCM4 and 10 molecules in the GEPIA database. We used the non-log scale for calculation and the log-scale axis for visualization. Results showed that the forecasts for both database sites were consistent (Figure 4B).

MCM4 inhibition suppressed GC cells growth

In order to verify the hypothesis, we explored the expression of MCM4 in GC tissues, results showed that MCM4 expression was upregulated in GC tissues compared with adjacent non-tumor tissues (Figure 5A and 5B). High MCM4 expression was significantly correlated with advanced TNM stage and lymph node metastasis (Figure 5C and 5D).

Next, we explored the functions of MCM4 in GC progression, si-MCM4 was transfected into MGC-803 and BGC-823 cells, and the transfection efficiency was determined by qRT-PCR (Figure 6A). MTS assays suggested that the proliferation ability was obviously inhibited in GC cells transfected with si-MCM4 compared to si-NC group (Figure 6B). Flow cytometry assay further confirmed that knockdown of MCM4 could arrest GC cells cycle in G0/G1 phase (Figure 6C). These data suggested that MCM4 might act as an oncogene in GC progression.

Discussion

Gastric cancer (GC) mainly develops from the innermost lining of the stomach, presenting the highest mortality rates among all digestive tract malignancies. This is mainly due to chemotherapy resistance and distant metastasis [24]. It is important to illustrate the pathogenesis of GC and discover novel prognostic strategies, early diagnostic tools, and effective therapeutic approaches.

MCM4 is one of the MCM proteins composing the pre-replicative complex that binds to replication origins in the G1 phase of the cell cycle. It is essential for the initiation of DNA replication [25]. Recent studies have shown that MCM4 might play important roles in tumor pro-
MCM4 expression in GC
MCM4 expression in GC

For example, Huang et al. showed that MCM4 was increased in esophageal cancer and associated with advanced pathological stage in patients from Southern China [26]. Gan et al. found that MCM4 was increased in cervical squamous cell carcinomas and associated with advanced clinical features [27]. Kikuchi et al. showed that MCM4 acted as a marker for proliferation and clinical and clinicopathological significance in non-small cell lung cancer [28]. However, information is limited regarding the roles of MCM4 in GC.

In the present study, we found MCM4 mRNA expression was significantly increased in gastric cancer based on the ONCOMINE datasets. Next, we analyzed MCM4 protein expression in clinical specimens from the human protein atlas (www.proteinatlas.org). Results showed that MCM4 protein had a strong positive expression in GC tissues, and a weak expression in normal gastric tissues. These findings indicated that MCM4 might act as an oncogene in GC progression.

To further explore the potential prognostic value of MCM4 in GC, the correlation between MCM4 expression and OS in GC patients was analyzed by KM plotter analysis. Results showed that high transcriptions of MCM4 was associated with poor OS in all GC patients, no matter the type, HER2 status or clinical stage. Next, STRING database showed that 10 candidate mRNAs which might interacted with MCM4, which was further forecast in the GEPIA database.

Figure 4. Network prediction and annotation of MCM4 with other molecules. A. 10 candidate mRNAs which might interacted with MCM4 in GC. B. The GEPIA database further confirmed the interaction between MCM4 and 10 candidate mRNAs. TPM, transcripts per million.

Figure 5. MCM4 was upregulated in GC tissues. A, B. MCM4 expression was upregulated in GC tissues. C, D. High MCM4 expression was associated with advanced TNM stage and lymph node metastasis upregulated in GC tissues. *P < 0.05.
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Figure 6. MCM4 inhibition suppressed GC cells proliferation in vivo. A. MCM4 expression levels in GC cells transfected with si-MCM4 and si-NC. B. MTS assays showed that the proliferation ability was obviously inhibited in GC cells transfected si-MCM4 compared to si-NC group. C. Flow cytometry assays showed that knockdown of MCM4 arrested GC cells in G0/G1 phase. *P < 0.05.
In order to verify the hypothesis, we explored the expression of MCM4 in GC tissues. Results showed that MCM4 expression in GC tissues was upregulated and associated with advanced TNM stage and lymph node metastasis of GC patients. In vitro function assays, we showed that MCM4 inhibition could significantly reduce GC cells proliferation and arrest GC cells in G0/G1 phase. Taken together, results indicate that MCM4 may play important roles in GC progression.

Conclusion

In summary, our results showed that MCM4 is distinctly high-expressed and predict poor overall survival in GC patients. Decreased MCM4 expression significantly suppressed GC cells proliferation both in vitro and in vivo. Thus, these results provided a better understanding of the heterogeneity and complexity in the molecular biology of GC, which provided potential new strategies for the therapy of GC.

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Disclosure of conflict of interest

None.

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References

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