MCM7 amplification and overexpression promote cell proliferation, colony formation and migration in esophageal squamous cell carcinoma by activating the AKT1/mTOR signaling pathway

YUN-TAN QIU*, WEN-JUN WANG*, BING ZHANG*, LI-LI MEI and ZHI-ZHOU SHI

Faculty of Medicine, Kunming University of Science and Technology, Kunming, Yunnan 650500, P.R. China

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Abstract. The roles and mechanisms of mini-chromosome maintenance complex component 7 (MCM7) amplification and overexpression in esophageal carcinogenesis were investigated. By analyzing the TCGA datasets, we found that MCM7 was amplified in approximately 12% of esophageal squamous cell carcinomas (ESCCs), and in more than 4% of head and neck squamous cell carcinomas and stomach carcinomas. Overexpression of MCM7 was further verified in three independent GEO datasets of esophageal cancer. Knockdown of MCM7 using two siRNAs significantly inhibited cell proliferation, colony formation and migration of KYSE510 and EC9706 cells *in vitro*. Noteworthy, we further found that silencing of MCM7 suppressed the phosphorylation of AKT1 and mTOR both in KYSE510 and EC9706 cells, and reduced the cell cycle regulatory proteins cyclin D1, cyclin E2 and CDK2. Taken together, our findings suggested that MCM7 promoted tumor cell proliferation, colony formation and migration of ESCC cells via activating AKT1/mTOR signaling pathway.

Introduction

Esophageal cancer is the sixth leading cause of cancer-related death in the world and the fourth leading cause of cancer-related death in China (1). There are two types of esophageal cancer, squamous cell carcinoma (SCC) and adenocarcinoma (AC), and most Chinese esophageal cancers are ESCCs (2). Although diagnostic methods and cancer treatments have improved in recent years, the prognosis is still poor because of widespread lymph node metastasis and relatively frequent distant metastasis. Therefore, understanding the mechanism of esophageal carcinogenesis will lay the foundations for improving clinical management and outcomes.

Mini-chromosome maintenance protein complex (MCM) is a eukaryotic DNA helicase complex required for initiation of DNA replication. The MCM proteins include six members, MCM2 to MCM7, and are considered as molecular markers of proliferation in several types of cancer (3-6). MCM7 is a critical component of DNA replication licensing complex, and is overexpressed in multiple human malignancies including hepatocellular carcinoma, head and neck squamous cell carcinoma, prostate carcinoma and esophageal squamous cell carcinoma (7-10). Kim *et al* found that upregulation of MCM7 was associated with cisplatin resistance in bladder cancer (11). In liver cancer MHCC-97 cells, silence of MCM7 dramatically reduced the cell proliferation, migration, invasion and increased the apoptotic cells (12).

Our previous and other studies found that MCM7 was amplified and overexpressed in ESCC, and overexpression of MCM7 was significantly linked with poor prognosis (7,13,14), however the roles and mechanisms of MCM7 amplification and overexpression in ESCC were largely unclear. In the present study, we revealed that MCM7 promoted tumor cell proliferation, colony formation and migration of ESCC cells via inhibiting the AKT1/mTOR signaling pathway.

Materials and methods

TCGA and GEO datasets. Genomic and expression data of MCM7 are publically available from the Cancer Genome Atlas and the NCBI Gene Expression Omnibus. The copy number alterations and mutations of TCGA datasets were analyzed by Cbioportal (www.cbioportal.org). For GSE datasets of GSE20347, GSE38129 and GSE29001, the mRNA expression levels are detected by microarray, and the difference of MCM7 between ESCC tissues and paracancerous tissues was analyzed using the paired t-test by SPSS 19.

Cell culture. The human esophageal cancer cell lines were supplied by Peking Union Medical College and Chinese

Correspondence to: Professor Zhi-Zhou Shi, Faculty of Medicine, Kunming University of Science and Technology, 727 Jingming South Road, Kunming, Yunnan 650500, P.R. China
E-mail: zhizhoushi@126.com

*Contributed equally

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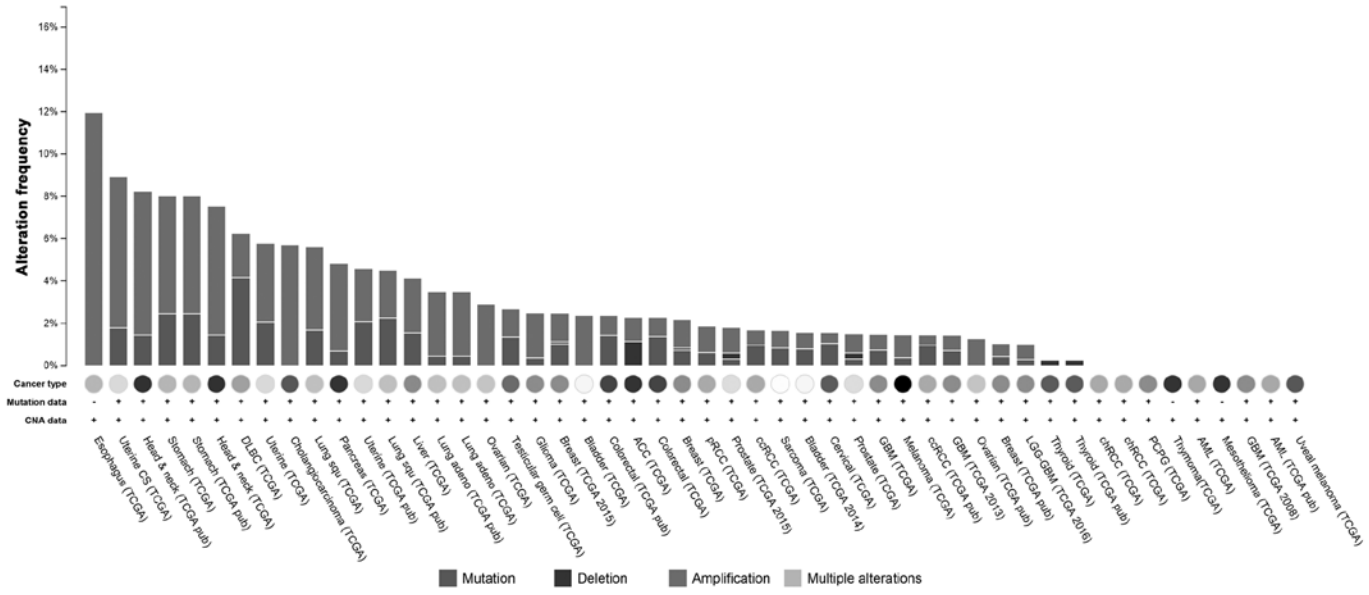

Figure 1. Amplification of MCM7 in many types of cancer in TCGA data. The figure was ploted using Cbioportal website and depicted the genomic aberration frequency. Panel shows the frequency and type of alterations for each analyzed study. The x-axis shows the types of cancer, availability of mutation and copy number variation data, and the study abbreviation.

Academy of Medical Sciences. All cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin at 37˚C in a humidified incubator containing 5% carbon dioxide.

siRNAs and transfection. The synthetic negative control siRNA, MCM7 siRNA-1 and MCM7 siRNA-2 were purchased from Shanghai Gene Pharma Co. Ltd. The ESCC cell lines were transiently transfected using Lipofectamine® 2000 Transfection Reagent from Invitrogen according to the manufacturer's protocol. The sequences of negative control siRNA, MCM7 siRNA-1 and MCM7 siRNA-2 were as follows: Negative control siRNA: 5'-UUCUCCGAACGUGUCACGUTT-3'; MCM7 siRNA-1: 5'-ATCGGATTGTGAAGATGAA-3'; MCM7 siRNA-2: 5'-AAGAUGUCCUGGACGUUUACA-3'.

Total RNA extraction and real-time PCR assay. Total RNA was isolated from cancer cells using the RNeasy mini kit as described by the manufacturer (Qiagen, Hilden, Germany) and used for Real-time PCR assay.

Real-time PCR was used to detect the mRNA expression levels of cyclin D1, cyclin E2 and CDK2. The PCR reactions were performed in a total volume of 20 μl, including 10 μl of 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK), 2 μl of cDNA (5 ng/μl) and 1 μl of primer mix (10 μM each). PCR amplification and detection were performed in a LightCycler 480 II (Roche Applied Science) as follows: an initial denaturation at 95˚C for 10 min; 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The relative gene expression was calculated using the comparative CT method. The gene expression of the target gene were normalized to an endogenous reference (GAPDH), and relative to the calibrator were given by the formula 2-ΔΔCt. ΔCT was calculated by subtracting the average GAPDH CT from the average CT of the gene of interest. The ratio defines the level of relative expression of the target gene to that of GAPDH. The primers were as follows: Cyclin D1 forward primer, 5'-ACGCTTACCTCAACCATCCTG-3'; Cyclin D1 reverse primer, 5'-GGCCTCTCGATACACACAACA-3'; Cyclin E2 forward primer, 5'-GCCCGGCCTATATATTGGGTT-3'; Cyclin E2 reverse primer, 5'-AACGGCTACTTCGTCT TGACA-3'; CDK2 forward primer, 5'-TCTTTGCTGAG ATGGTGACTCG-3'; CDK2 reverse primer, 5'-TCTTCA TCCAGGGGAGGTACA-3'; GAPDH forward primer, 5'-AAATCCCATCACCATCTTCCAG-3'; GAPDH reverse primer, 5'-GAGTCCTTCCACGATACCAAAGTTG-3'.

Western blot assay. Cells were lysed in the lysis buffer (20 mM Tris, 2 mM EDTA, 50 mM 2-mecaptoethanol, 10% glycerol, pH 7.4). The homogenates were placed on ice for 30 min and centrifuged at 12,000 x g for 15 min at 4˚C. Afterward, the protein concentrations of the lysates were determined using a Protein Assay kit (Bio-Rad, Richmond, CA, USA). Equal amounts of total proteins were loaded onto a 10% gradient polyacrylamide gel, electrophoretically transferred to polyvinylidene difluoride membrane, and then blocked with 10% non-fat milk for 2 h at room temperature. The membranes were incubated with specific primary antibodies overnight at 4˚C and probed with corresponding secondary antibodies for 1 h at room temperature. The protein bands were visualized using ECL Blotting Detection Reagents (Applygen, Beijing, China). The primary antibodies were as follows: MCM7 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-AKT1 (1:1000; Santa Cruz Biotechnology), AKT1 (1:1000; Santa Cruz Biotechnology), p-mTOR (1:1000; Santa Cruz Biotechnology), mTOR (1:1000; Santa Cruz Biotechnology) and β-actin (1:5000; Santa Cruz Biotechnology).

Cell proliferation assay. The Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) was performed to quantify the proliferation of KYSE510 and EC9706 cells. Cells were cultured at 1000/well in 96-well plates. After incubated for 24,

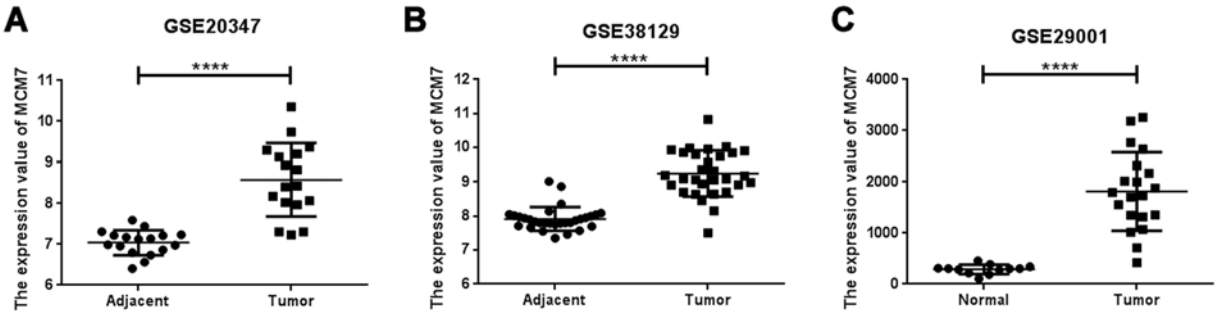

Figure 2. MCM7 is overexpressed in ESCC by analyzing GSE datasets. The differences of expression levels of MCM7 in tumor tissues and para-cancerous tissues in the datasets of GSE20347 (A), GSE38129 (B) and GSE29001 (C) were analyzed by using Student's two-tailed t-test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

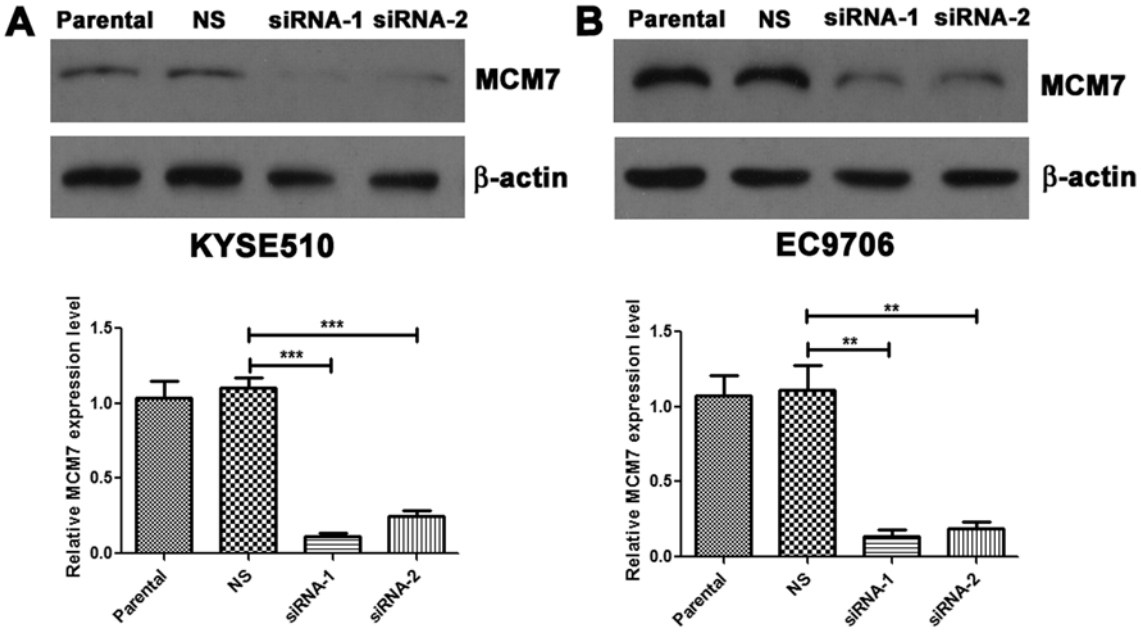

Figure 3. The RNAi efficiency of siRNAs targeting MCM7 in KYSE510 and EC9706 cells. The RNAi efficiency of siRNA-1 and siRNA-2 targeting MCM7 in KYSE510 (A) and EC9706 (B) cells was detected by western blot assay. Data are presented as mean ± SEM of n=3 independent experiments. The differences between MCM7 siRNA group and negative control group were analyzed by using Student's two-tailed t-test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

48, 72, 84, 96, or 120 h, 10 μl of CCK-8 was added to each well and incubated for 1 h. The absorbance of each well was read at 450 nm. Three independent experiments were performed.

Colony formation assay. For each group, 5000 cells were plated in 6-well plate. After cultured for 10 days, the cells were washed with PBS, fixed with methanol and 0.1% crystal violet. Then, the colonies were counted and photographed. Three independent experiments were performed.

Transwell assay. The migration assay was performed on Transwell plates. For cell migration assay, $1x10^5$ cells were seeded on a polycarbonate membrane insert in a Transwell apparatus (Costar, Cambridge, MA, USA) and cultured in RPMI-1640 without serum. RPMI-1640 containing 20% fetal bovine serum was added to the lower chamber. After incubation for 24 h at 37˚C in a CO_2 incubator, the insert was washed with PBS, and cells on the top surface of the insert were removed by wiping with a cotton swab. Cells that migrated to the bottom surface of the insert were fixed with methanol, stained with 0.4% crystal violet, and counted in five random fields at x200.

Statistical analysis. The data were analyzed by Student's t-test and one-way analysis of variance using the SPSS and Graphpad Prism 5.0. P<0.05 was considered to indicate a statistical significant difference.

Results

MCM7 is amplified and overexpressed in many types of cancer including ESCC. The datasets of The Cancer Genome Atlas (TCGA) showed that MCM7 was amplified in approximately 12% of ESCCs, and in >4% of head and neck squamous cell carcinomas and stomach carcinomas, and the amplification was the dominant form of changes in DNA level; however in diffuse large B-cell lymphoma (DLBC), mutation was more frequent than amplification (Fig. 1). MCM7 was overexpressed

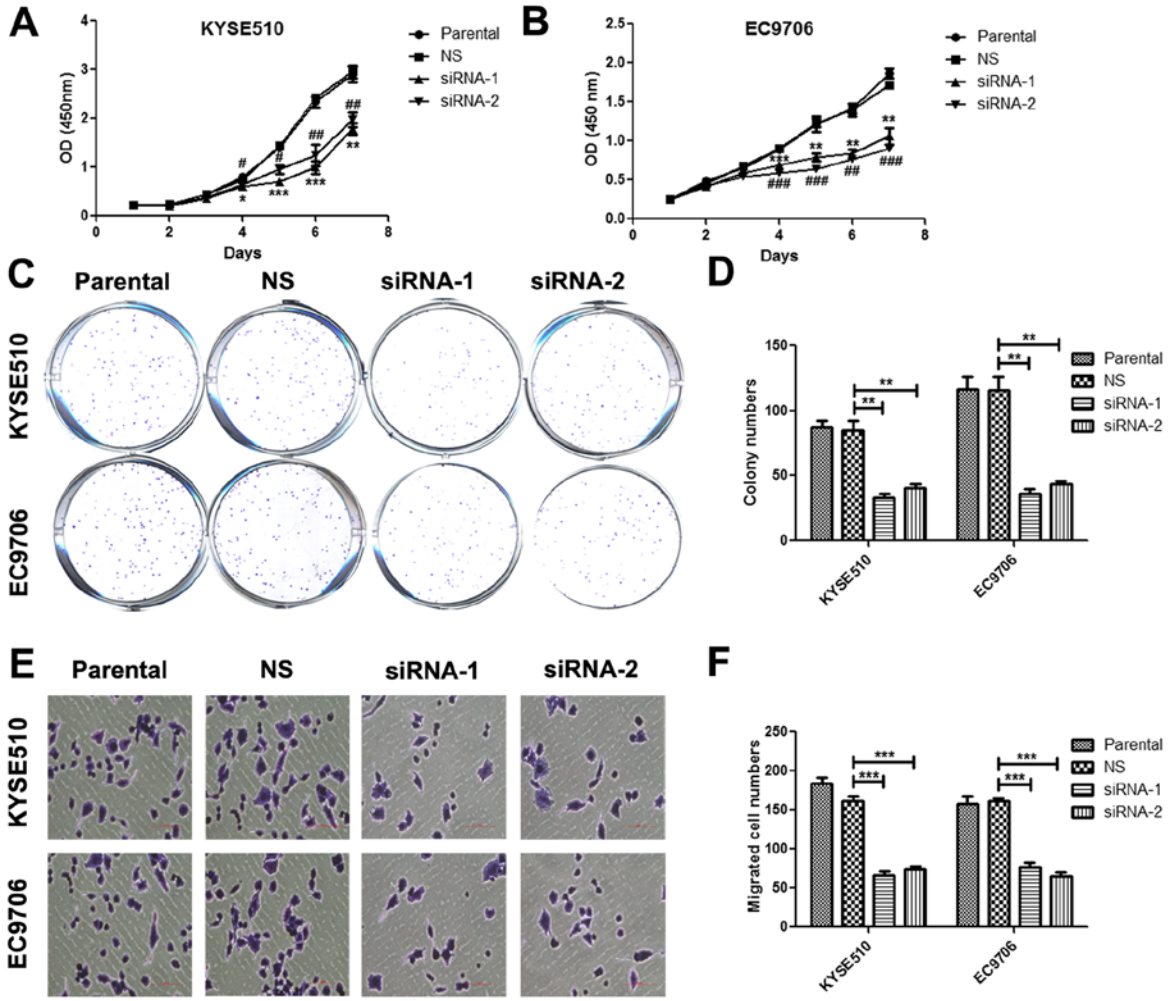

Figure 4. Knockdown of MCM7 suppresses cell proliferation, colony formation and migration of KYSE510 and EC9706 cells. Effects of MCM7 siRNAs on the cell proliferation of KYSE510 (A) and EC9706 (B) cells were detected by Cell Counting Kit-8. (C and D) Effects of MCM7 siRNAs on the colony formation of KYSE510 and EC9706 cells was measured by crystal violet staining. (E and F) Effects of MCM7 siRNAs on the migration of KYSE510 and EC9706 cells were detected by Transwell assay. Representative images are shown. Data are presented as mean ± SEM of n=3 independent experiments. The differences between MCM7 siRNAs group and negative control group were analyzed by using Student's two-tailed t-test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

in ESCC tissues in the datasets of GSE20347, GSE38129 and GSE29001 (Fig. 2).

Knockdown of MCM7 suppresses the proliferation, colony formation and migration of ESCC cells. In order to explore the tumorigenic roles and mechanisms of MCM7 in esophageal carcinogenesis, we selected cell lines KYSE510 and EC9706 with higher MCM7 expression for further study.

siRNAs were used to knock down the MCM7 expression in KYSE510 and EC9706 cells, and the RNAi efficiency was determined by western blot assay (Fig. 3A and B). Using CCK-8 proliferation assay, we found that silence of MCM7 significantly inhibited cell proliferation compared with non-silencing group, and there was significant suppression at days 4, 5, 6 and 7 both in KYSE510 and EC9706 cells (Fig. 4A and B). In colony formation assay, we observed that colony numbers were significantly lower in MCM7 siRNA-1 and siRNA-2 transfected cells than in the control group both in KYSE510 and EC9706 cells (Fig. 4C and D). By Transwell assay, we revealed that silence of MCM7 significantly inhibited the migration of KYSE510 and EC9706 cells (Fig. 4E and F).

Knockdown of MCM7 suppressed the AKT1/mTOR signaling pathway in ESCC. Many studies showed that activated AKT1/mTOR signaling pathway promoted cancer cell proliferation, epithelial-mesenchymal transition (EMT), tumor metastasis and invasion (15-20). Noteworthy, our study revealed that silencing MCM7 significantly inhibited the phosphorylation of AKT1 and mTOR (Fig. 5). In addition, knockdown of MCM7 reduced the cell cycle regulatory genes cyclin D1, cyclin E2 and CDK2 in mRNA expression levels (Fig. 6). These results indicated that MCM7 amplification and overexpression promoted cell proliferation, colony formation and migration via activating the AKT1/mTOR signaling pathway.

Discussion

Genomic aberrations can contribute to carcinogenesis and tumor progression. In the past decades, the understanding of molecular pathogenesis of ESCC has developed, but is still limited. Thus, elucidation of the mechanism of esophageal carcinogenesis was very important for tumor diagnosis and

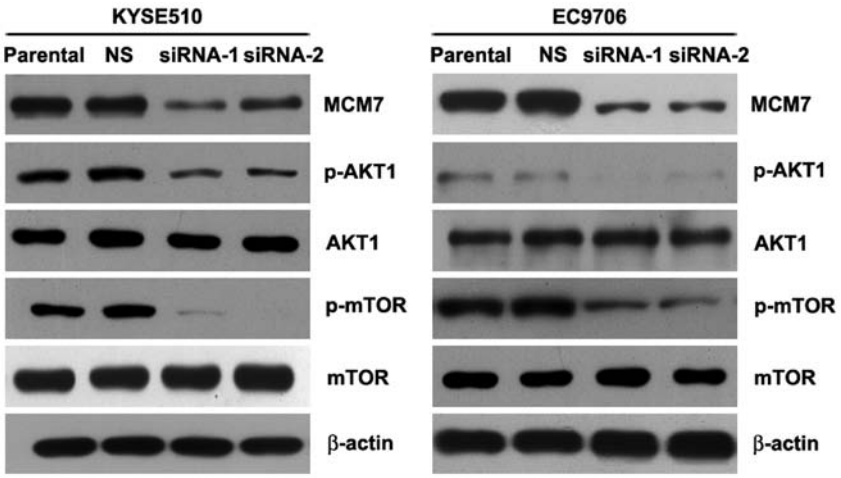

Figure 5. Knockdown of MCM7 inhibits phosphorylation of AKT1 and mTOR in KYSE510 and EC9706 cells. Protein levels of MCM7, p-AKT1, AKT1, p-mTOR, mTOR and β-actin in KYSE510 and EC9706 cells. Cells were transfected with negative control siRNA, MCM7 siRNA-1 and siRNA-2 for 48 h. All the protein levels were determined by western blotting. The experiment was repeated three times.

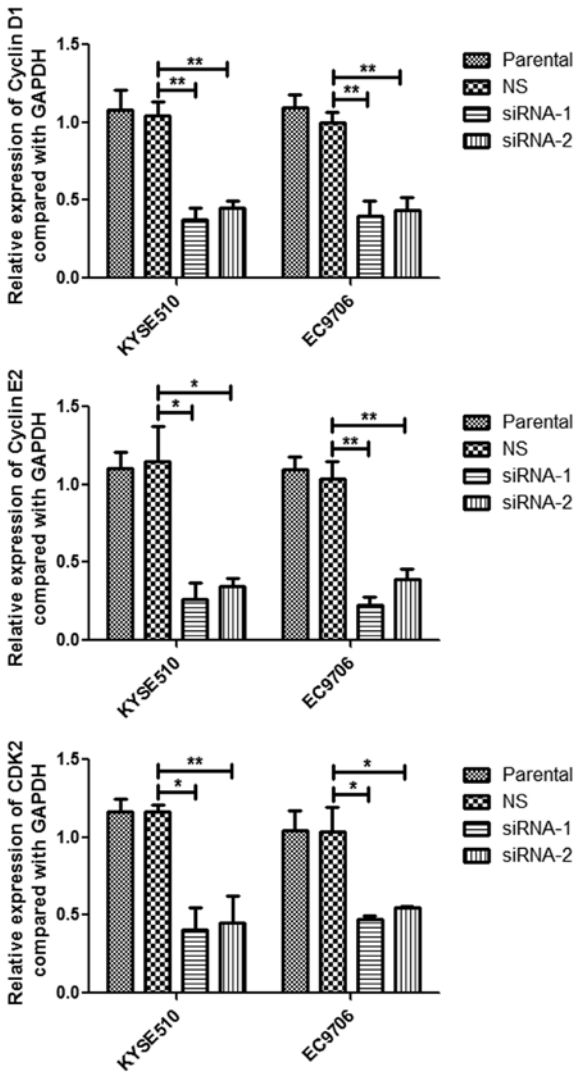

Figure 6. Knockdown of MCM7 reduces the mRNA expression levels of cyclin D1, cyclin E2 and CDK2. Cells were transfected with negative control siRNA, MCM7 siRNA-1 and siRNA-2 for 48 h. All the mRNA levels were determined by real-time PCR normalized with GAPDH. Data are presented as mean ± SEM, and the differences between MCM7 siRNA group and negative control group were analyzed by using Student's two-tailed t-test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

therapy. Recently, it was shown that MCM proteins were overexpressed in several types of tumors, and high expression levels of MCM proteins including MCM2, MCM3 and MCM7 were positively correlated with Ki-67 positive staining in Laryngeal squamous cell carcinoma (LSCC) (5).

MCM7 is a critical DNA replication licensing factor in both yeast and xenopus oocytes, and serves as a co-transcriptional and co-translational enhancing factor of androgen receptor, which regulates cell growth and proliferation (21). Increased MCM7 expression is common in various human cancers including esophageal squamous cell carcinoma, prostate cancer and pancreatic cancer (7,10,22,23), and MCM7 has been considered as a tumorigenesis-related gene (24). In pituitary adenoma, diffuse-type primary gastric adenocarcinoma and colorectal cancer, the patients with MCM7 overexpression had a shorter recurrence/progression-free survival respectively (25,26). MCM7 promotes tumor cell proliferation and invasion in papillary urothelial neoplasia and liver cancer (27), and depletion of MCM7 inhibits glioblastoma multiforme tumor growth *in vivo* (28). In ESCC, G9a and MCM7 overexpression levels were correlated with poor prognosis (7), however, the roles and mechanisms of MCM7 amplification and overexpression in ESCC were largely unknown. Our results showed that MCM7 promoted tumor cell proliferation, colony formation and migration of ESCC cells.

AKT, a serine/threonine kinase, has a wide tissue distribution and regulates many processes including cell metabolism, proliferation, survival and tumor growth (29). Notably, AKT has been considered as a critical oncogene, and it can activate downstream signaling pathways through phosphorylation of a plethora of AKT substrates (30). In recent studies, the phosphorylation of AKT was shown correlated with poor prognosis in ESCC (31,32). AKT was responsible for the cisplatin resistance, and could promote metastasis of ESCC by targeting epithelial-mesenchymal transition (33-35). mTOR activation was able to also prcomote the cell proliferation and tumor progression of ESCC (36,37). However, the direct relationship between AKT/mTOR and MCM7 has not been reported. Our findings further revealed that knockdown

of MCM7 significantly inhibited the phosphorylation of AKT and mTOR.

In summary, our data revealed that MCM7 promoted tumor cell proliferation, colony formation and migration via activating the AKT1/mTOR signaling pathway. Future studies should focus on how MCM7 regulates the AKT1/mTOR signaling pathway, and explore the antitumor activity of MCM7 silencing in an animal model.

Acknowledgements

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