Minichromosome maintenance (MCM) protein 4 overexpression is a potential prognostic marker for laryngeal squamous cell carcinoma

Jianfeng Han[1,2], Meng Lian[1], Jugao Fang[1], Honggang Liu[3], Ru Wang[1], Jie Zhai[1], Yi Fan Yang[1], Ling Fen[1], Qian Shi[1], Hong Zhi Ma[1], Juan Sun[4], Zhihong Ma[5]

[1]Department of Otorhinolaryngology, Head and Neck Surgery, Beijing Tongren Hospital, Capital Medical University, Beijing, 100730, China; [2]Department of Otorhinolaryngology, Head and Neck Surgery, Inner Mongolia People's Hospital, Hohhot, 010017, China; [3]Beijing Key Laboratory of Head and Neck Molecular Diagnostic Pathology, Beijing, 100730, China; [4]Inner Mongolia Medical University Affiliated Hospital, Otolaryngology Department, Hohhot, 010050, China; [5]Department of Otolaryngology, Chengde Central Hospital, Chengde, Hebei, 067000, China

Summary

Purpose: The minichromosomal maintenance (MCM) proteins are involved in the initiation and DNA replication. The role of MCM4 remains to be elucidated. The purpose of this study was to investigate the effects of MCM4 in laryngeal squamous cell carcinoma (LSCC) cell growth and apoptosis.

Methods: LSCC cell line UMSCC 5 was used in this study. The small interfering RNA (siRNA) of MCM 4 gene was used to identify the effects of MCM4 on the proliferation and apoptosis using methylimidazole tetrazolium (MTT) assay and flow-cytometry, respectively. Confirmed LSCC and adjacent non-tumor tissues were collected from 34 patients who were willing to participate in the study, from 2010 through 2015, from 163 patients undergoing treatment in the Department of Otorhinolaryngology/Head and Neck Surgery of Beijing Tongren Hospital in Capital Medical University of P.R. China. Immunohistochemical staining of MCM4 expression in the resected tissues was performed to analyze the correlation between its expression and the clinicopathological characteristics.

Results: The results showed that siRNA of MCM4 could significantly inhibit LSCC cell line UMSCC 5 proliferation and induce apoptosis. MCM4 mRNA was higher expressed in carcinoma tissues than in adjacent normal tissues. MCM4 expression was correlated with male gender, smoking history and poor differentiation.

Conclusions: We noticed a significant role for MCM4 overexpression in human LSCC tissues and their corresponding adjacent non-neoplastic tissues and found that siRNA of MCM4 can significantly decrease the proliferation of cancer cells. It is suggested that MCM4 profiling could potentially be used to predict response to treatment and prognosis in LSCC.

Key words: laryngeal squamous cell carcinoma, minichromosomal maintenance protein 4 (MCM4), siRNA

Introduction

Laryngeal carcinoma is one of the common malignancies of head and neck around the world, accounting for 1-2.5% of all malignancies. Around 95% of the cases are LSCC [1-3]. LSCC comprises several groups of tumors arising from the epithelium of the upper respiratory tract and salivary and thyroid glands. Despite lots of advances in early detection and treatment of LSCC, the prognosis of LSCC remains poor, and 5-year survival is around 60% [4]. The routine treatment of LSCC comprises surgery, radiation therapy with induction chemotherapy and a combination of modalities, but the adverse effects of those treatments can create devastating damage on swallowing or

speaking functions [3]. Although computerized tomography (CT) and magnetic resonance imaging (MRI) are basic tools in the diagnosis of LSCC, sensitive biomarkers for LSCC are still lacking. Satisfactory diagnosis and appropriate therapy are usually delayed in laryngeal cancer. Traore et al. reported that less than 30% of laryngeal cancer patients were diagnosed and underwent surgery due to lack of accurate diagnosis in early disease stage [5]. Thus, better understanding of LSCC and identification of new molecular biomarkers are pivotal to improve diagnosis and treatment strategies for this cancer.

The MCM protein complex, which comprises 6 groups of proteins, is involved in the initiation and DNA replication [6]. The MCM helicase plays an important role on maintaining genome stability at the replication phase. MCM4, one of the MCM family members, is essential for the initiation of eukaryotic genome replication and may be involved in the formation of replication forks and in the recruitment of other DNA replication-related proteins [7,8]. Deregulation of MCM4 was reported in several cancers, which could serve as a potential promising prognostic marker of LSCC [9,10].

RNA interference (RNAi) has been studied as a powerful tool to suppress the functions of specific genes by triggering post-transcriptional degradation of homologous transcripts [11,12]. Lentiviruses vectors are useful tools to deliver exogenous genes with stable transgene expression *in vitro* and *in vivo* [13]. In this study, we used the lentivirus vector system to deliver a specially designed siRNA of MCM4 gene into the laryngeal cancer cell line UMSCC5, to detect the effects of siRNA MCM4 on cell proliferation and apoptosis. Also, we evaluated the MCM4 expression in 163 cases of surgically resected LSCC tissues to correlate clinicopathological characteristics.

Methods

Patient samples

LSCC and adjacent non-tumor tissues were collected from 34 patients with confirmed LSCC who were willing to participate in the study, from 163 patients undergoing treatment in the department of Otorhinolaryngology/Head and Neck Surgery of Beijing Tongren Hospital in Capital Medical University from 2010 to 2015. This study was approved by the ethics committee of Beijing Tongren Hospital in Capital Medical University. Signed written informed consents were obtained from all participants before the start of the study.

Cell culture and lentivirus siRNA gene transfection

Laryngeal squamous cell line UMSS5 was obtained from University of Michigan (USA). Cells were cultured in the high glucose DMEM medium (Gibco) with 10% fetal bovine serum (FBS), and grown in an incubator at 37°C and 5% CO_2 atmosphere. siRNA of MCM4 (21-bp) lentivirus gene transfer vector was purchased from Genechem Co., Ltd (Shanghai, China). Cells were seeded into a 96-well plate with cell density 4×10^3/well and incubated for 24 hrs. Then, cells were transfected with siRNA for 48 and 72 hrs following the instructions of the manufacturer. Cells without siRNA transfection served as negative control group.

Cell proliferation assay

The proliferation of UMSCC5 cells was detected using cell proliferation reagent kit I (MTT). Cells were transfected with siRNA were plated into a 96-well plate and incubated for 48 and 72 hrs. In each well 200 μL MTT solution (5 mg/mL) was added and the cells were incubated for 4 hrs at 37°C. DMSO 150 μL/well was added and incubated for 5 min and the optical density (OD) values were assessed at 490 nm using Versamzx microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometric analysis of apoptosis

The FITC Annexin V/PI apoptosis detection kit (BD Biosciences, San Diego, USA) was used to measure cell apoptosis. The UMSCC5 cells were transfected with siRNA and incubated for 48 and 72 hrs in 6-well plate. Control cells were not tranfected with siRNA. Then, the cells were washed twice with cold PBS, and harvested by trypsinization. Following this, the cells were double-stained with annexin V-FITC and PI for 15 min at 37°C. Then, the cells were assessed with flow cytometer (CyAn ADP9, Beckman Coulter, Fullerton, USA). All cells were divided into living cells, early apoptotic cells and late apoptotic cells. The percentage of cells with apoptotic nuclei (percent apoptosis) was calculated.

Quantitative real-time PCR assay

Quantitative real-time PCR was performed to evaluate the expression level of MCM4 and β-actin of LSCC tissues. The patients' tissues were collected during surgery. We matched the expression comparing the expression pattern between laryngeal cancer tissues and the expression status of genes from the adjacent mucosa (< 2 cm away from the carcinomas).

The frozen tissues were minced with a homogenizer. For RNA isolation, total RNA was extracted with Trizol reagent (Gibco BRL, NY, USA), the concentration and purity of RNA were determined at 260/280 nm using a nanodrop spectrophotometer. One μg of each isolated RNA was subjected to reverse transcription of cDNA synthesis, conducted in a 14 μL reaction buffer, containing 1 μL reverse transcriptase (50 U) and 1 μL primer, according to manufacturer's instructions (Takara, Japan). With the obtained for cDNA as a template, the relative expression levels of MCM4 and β-actin from patients receiving experimental treatment were determined by PCR. The sequence of the primers for RT-PCR were as follows: MCM4, Forward, 5'-GACCAGCTCATCACCATCAG-3'; and Re-

verse, 5'-TGCTTGTCAGAGAAGAGGGA-3'; β-actin Forward, 5'-CACTCTTCCAGCCTTCCTTC-3'; and Reverse, 5'-TGATCTTCATTGTGCTGGGT-3'. Each 20 μL reaction system comprised 2 μL of cDNA, 10 μL SYBR Premix Ex Taq II, 10 μmol/L of both sense and antisense primers. All data for each sample were measured in triplicate and using $2^{-\Delta\Delta Ct}$ method.

Immunohistochemical analysis

MCM4 expression was analyzed by immunohistochemistry as described previously [14]. The labeled streptavidin-biotin-peroxidase method was used on 4-μm sections of formalin-fixed, paraffin-embedded tissues after deparaffinization. Briefly, deparaffinized tissue sections were treated with an autoclave in 10 mM citrate buffer (pH 6.0) for 20 min at 121 °C to assess antigenicity. Sections were then immersed in methanol containing 1.5% hydrogen peroxide for 20 min to block endogenous peroxidase activity, and were further incubated with normal rabbit serum to block nonspecific antibody binding sites. Sections reacted consecutively with anti-MCM4 antibody produced in mouse purified immunoglobulin (Sigma–Aldrich SAB1406110-50UG Lot:CC031), at a dilution of 1:50 at 4°C overnight. Immunostaining was performed by the streptavidin-biotin-peroxidase method with 3,3-diaminobenzidine as a chromogen (SAB-PO kit; Nichirei, Tokyo, Japan). Hematoxylin solution was used for counterstaining. Blocks of UMSCC 5 cells treated with or without MCM4 siRNA acted as positive or negative controls. Tumor cells were considered MCM4 positive if any nuclear staining was present.

MCM4 labelling index (LI) in carcinoma cells (%) was defined as the percentage of tumor cells displaying nuclear immunoreactivity and was calculated by the positive staining of MCM4 cells in 500 tumor cells in each sample. The tumors were divided into a group with MCM4 LI < 50%, and another LI group with > 50%. To identify if MCM4 could serve as a biomarker of LSCC, the relationship of MCM4 expression levels, correlated with clinicopathological features, were studied. The immunohistochemically staining sections were reviewed and scored by two independent investigators using Olympus microscope. Sections stained for MCM4 protein expression were scored from 0 to 4: 0, negative (no staining); 1, weakly positive (positive cells ≤ 10%); 2, positive (positive cells > 10% but ≤ 25%); 3, strongly positive (positive cells > 25% but ≤ 50%); and 4, very strongly positive (positive cells > 50%). And last, we defined 0 as negative and 1 as positive to avoid a zero number in one or more groups.

Statistics

Data are expressed as mean±SD. Statistical differences were evaluated by SPSS 19.0 software. Differences in expression levels of MCM4 mRNA and protein between tumor and corresponding adjacent non-neoplastic tissues in our cohort were analyzed using paired-*t* test.

P values less than 0.05 were considered as statistically significant.

Results

siRNA MCM4 suppresses cell proliferation and induces apoptosis

MTT assay was used to evaluate the effect of siRNA of MCM4 on LSCC cell line UMSCC5 on cell proliferation. The results showed that siRNA treatment could significantly suppress the cell proliferation compared with the control group (Figure 1) for over 72 hrs after transfection. When cells were transfected with siRNA, the viable cells were markedly reduced compared with control group (Figure 1). The inhibition rates were 27.23, 32.80, and 36.17% at 24, 48 and 72 hrs after transfection, respectively. The time-effect curves demonstrated that MCM4 could suppress the cell proliferation of UMSCC5 cell *in vitro*.
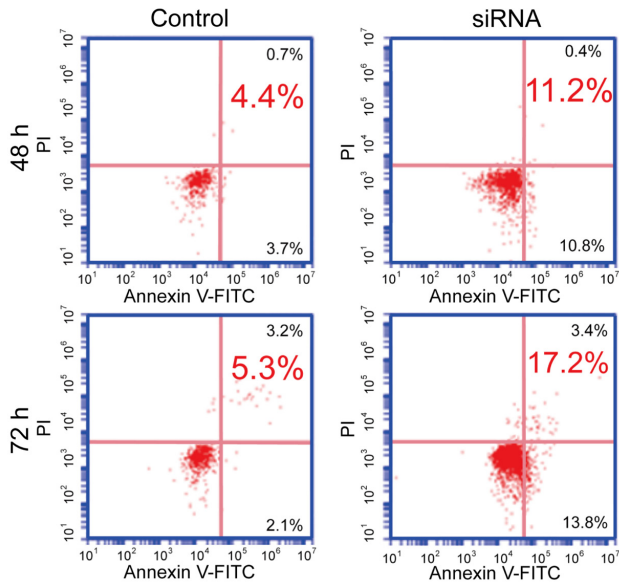
Flow cytometry was used to detect the cell apoptosis. The results demonstrated that siRNA treatment could increase cell apoptosis compared to control cells (Figure 2). After transfection, the apoptosis rates at 48 and 72 hrs were 11.2±0.2 and 17.2±0.6, respectively, which were significantly higher than that of the control groups. These results suggested that siRNA of MCM4 could inhibit LSCC cell line UMSCC5 proliferation and induce apoptosis.
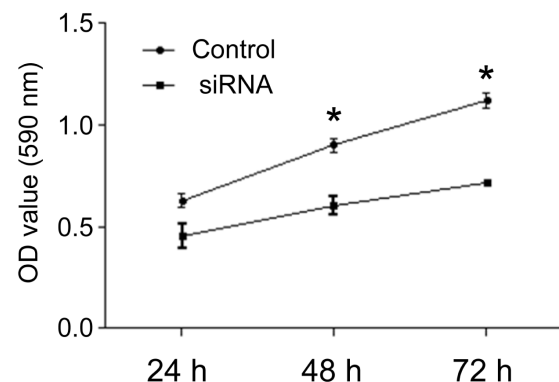

Figure 1. Effect of siRNA of MCM4 on cell proliferation of laryngeal squamous cell carcinoma cell line UMSCC5. MTT assay was performed to evaluate the proliferation at different time points. *p<0.05, compared with control group.

MCM4 gene expression analysis in LSCC

MCM4 are important for DNA replication in cells, restricting replication of once per cell cycle. To investigate whether MCM4 could distinguish LSCC from normal tissues, 34 cancer tissues and their adjacent non-neoplastic tissues were analyzed by qRT-PCR. mRNA of MCM4 expression showed statistically significant difference, higher than that of adjacent normal tissues (Table 1, p<0.05). MCM4 were upregulated in LSCC tissues.

Figure 2. Effect of siRNA of MCM4 on cell apoptosis of laryngeal squamous cell carcinoma cell line UMSCC5. Apoptosis rates were determined by flow cytometry using staining of Annexin V-FITC and PI. Flow cytometry was used to detect the cell apoptosis. Compared with control cells, siRNA treatment increased cell apoptosis. Apoptosis rates of cells with siRNA transfection at 48 and 72 hrs were 11.2±0.2 and 17.2±0.6, respectively, which were significantly higher than that of the control groups.
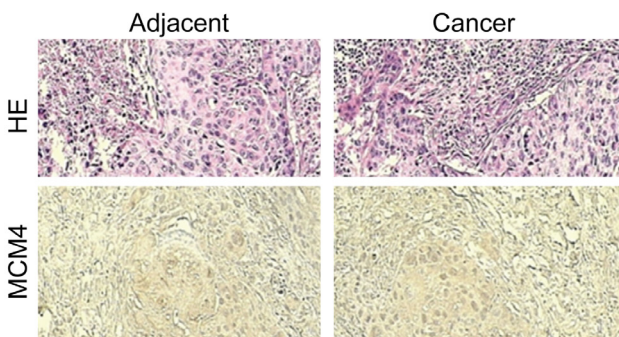
Figure 3. Hematoxylin-eosin (HE) staining and immunohistochemical staining of MCM4 in laryngeal squamous cell carcinoma tissues and the adjacent normal tissues (original magnification 100×). MCM4 was mainly expressed in nuclei; expression level of MCM4 protein was lower in adjacent normal tissues than in cancer tissues.

Immunohistochemical analysis

The results of tumor MCM4 immunohistochemical staining are shown in Figure 3. LSCC tissues showed abundant MCM4 expression in nuclei, while MCM4 protein expression was much lower in adjacent normal tissues compared with cancer tissues. These results were consistent with the gene expression results in LSCC tissues and adjacent normal tissues.

As shown in Table 2, high MCM4 expression was found in 27 LSCC. Gender (male) and smoking history were significantly correlated with LSCC,

Table 1. qRT-PCR analysis between laryngeal squamous cell carcinoma tissues and their corresponding adjacent non-neoplastic tissues

Group	n	MCM4	t	p value
Laryngeal squamous cell carcinoma	34	1.609±0.329	12.36	0.00
Adjacent nontumor tissues	34	0.986±0.037		

Table 2. Relationship between MCM4 expression and clinicopathogical charactersitics in 163 cases of laryngeal squamous cell carcinoma

Characteristics	MCM4 expression		
	High	Low	p value
Age (y)			0.9399
≤60	4	3	
>60	15	12	
Sex			<0.0001
Male	15	5	
Female	12	2	
Differentiation			0.4885
Good	1	2	
Moderate	11	5	
Poor	10	5	
pT classification			0.438
T1-T2	17	2	
T3-T4	12	3	
pN classification			0.8736
N0	15	3	
N1-3	13	3	
Smoking			0.0042
Smoker	26	4	
Non-smoker	1	3	

but there was no significant difference between MCM4 expression and age, pathological nodal stage (pN), histology and pathological TNM stage.

Discussion

In the current study, siRNA of MCM4 was used to evaluate the suppressed proliferation and inducible apoptosis effects on LSCC UMSCC5 cell line. MCM4 gene and protein were overexpressed in tumor tissues, showing their correlation with LSCC clinicopathological characteristics.

Given that MCM4 plays a critical role in the initiation and replication of DNA, its expression is considered to relate with cell proliferation [15,16]. MCM4 could stabilize the genome through its checkpoint independent role [17]. Overexpression or amplification of MCM4 genes were found in

several types of cancers and in mouse carcinoma models. It was reported that MCM4 and MCM7 displayed similar distribution with another proliferation biomarker, Ki67, however MCM4 proteins are more sensitive and accurate as proliferation markers in cancers [18,19]. In the present study, qRT-PCR results demonstrated that the MCM4 mRNA was much higher in carcinoma tissues than in adjacent normal tissues. These findings may characterize MCM4 as a sensitive marker of proliferation in various malignant tumors. To further confirm the functions of MCM4 on cell proliferation and apoptosis, RNA interference, a prevailing genetic tool for silencing gene expression, was performed in this study. Lentiviruses vector system as a feasible tool was used to deliver siRNA of MCM4 into LSCC UMSCC5 cells. The lentiviruses are a family of retroviruses that can be used to integrate the target siRNA into the genomes of dividing and nondividing cells. In the transfected cells exogenous genes can usually be stably expressed for a long time. The present results showed remarkable suppression effects on cell proliferation and upregulated apoptosis after siRNA transfection in MTT assay and flow cytometric analysis. LSCC UMSCC5 cells in the negative control group continued to grow and divide, with a 178.7% increase in cell viability at 72 hrs post-transfection. In contrast, UMSCC5 cells transfected with siRNA demonstrated loss of cell viability, with a 36.17% inhibition of cell proliferation at 72 hrs post-transfection. In this study, cells had not totally lost the ability to grow by siRNA. These results were consistent with Bailis study [19] who suggested that MCM4 was involved in the regulation process of cancer cell proliferation and apoptosis, probably through changing chromosome structure or cell cycle progression [20]. In previous studies, MCM4 knockdown in A549 cells induced G1 arrest and apoptosis [21], and in a mouse model a disrupted MCMC allele (MCM 4 -/-) induced lethal effects [22].

Our study found that MCM4 expression in LSCC was significantly associated with clinico-pathologic characteristics and with male gender and smoking. Kikuchi et al. also reported the association between MCM4 expression and male gender and smoking history [18]. Fujioka et al. found that MCM7 was significantly correlated with poor prognosis in lung cancer patients [23]. Taken together, siRNA of MCM4 could inhibit carcinoma cell growth and induce apoptosis and overexpression of MCM4 in LSCC carcinoma tissues. We believe that MCM4 could be a promising prognostic biomarker of LSCC.

Conclusion

Our results demonstrated that siRNA of MCM4 can suppress cell proliferation and apoptosis of LSCC UMSCC5 cells. It could be concluded that in the present study the increased expression of MCM4 might be associated with laryngeal cancer. MCM4 may be used as a prognostic marker for LSCC. In our future projects, we will increase the sample to confirm this conclusion.

Acknowledgements

Conflict of interests

The authors declare no confict of interests.

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