Histol Histopathol (2024) 39: 471-482

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE Open Access

Systematic analysis of expression and prognostic significance for MCM family in head and neck squamous cell carcinoma

Ercan Sun*, Lu Peng*, Zhe Liu*, Zeng Yan, Min Chen and Jun Zheng

The First Affiliated Hospital of Shihezi University, Shihezi, Xinjiang, China

*Ercan Sun, Lu Peng and Zhe Liu contributed equally

Summary. Background. Head and neck squamous cell carcinoma (HNSC) is a common malignant tumor in the world and has a poor prognosis. The family of minichromosome maintenance proteins (MCM) improves the stability of genome replication by inhibiting the rate of DNA replication in eukaryotic cells, thus, small changes in physiological MCM levels would increase the instability of gene replication and increase the incidence of tumor formation, most of which are significantly elevated in multiple cancers. However, the expression of different MCM families in HNSC and their prognostic value remain unclear.

Methods. ONCOMINE and GEPIA databases were used to analyze the expression of MCMs in HNSC. The Kaplan-Meier plotter database was used to identify molecules with prognostic values. We collected 77 HNSC tissues and 50 normal tissues to validate the results of the bioinformatics analysis by immunohistochemical staining.

Results. The expression of MCM3, MCM5 and MCM6 in mRNA and protein levels were higher in HNSC. Moreover, the increased expression of MCM3, MCM5 and MCM6 in mRNA and protein levels predicted better prognosis of HNSC patients. Furthermore, multivariate analysis showed that high expressions of MCM3, MCM5 and MCM6 in protein level may be independent prognostic factors for HNSC patients.

Conclusion. The results of this study indicated that MCM3, MCM5 and MCM6 play an important role in occurrence and development in HNSC and might be risk factors for the survival of HNSC patients.

Corresponding Author: Jun Zheng or Min Chen, The First Affiliated Hospital of Shihezi University, Shihezi, Xinjiang, China. e-mail: 1298083486@qq.com or 2921180391@qq.com

www.hh.um.es. DOI: 10.14670/HH-18-652

Key words: Head and neck squamous cell carcinoma, MCM family, Bioinformatics analysis, Biomarker, Prognostic value

Introduction

Head and neck squamous cell carcinoma (HNSC) is one of the most common malignancies, accounting for about 900,000 new cases and 500,000 deaths each year (Bray et al., 2018). Today, although there have been advances in follow-up treatments, such as surgery, radiation, and chemotherapy, there is a 5-year survival rate of less than 50% because many patients are diagnosed with advanced HNSC (Vigneswaran and Willians, 2014; Cohen et al., 2016). In recent years, tumor immunotherapy and targeted therapy have been widely used in the treatment of HNSC, becoming an important therapeutic approach (Zhang et al., 2020a,b). Therefore, we urgently need to find better molecular markers of HNSC to improve the diagnosis of HNSC patients, better predict the prognosis of patients, and provide a reasonable personalized treatment for patients.

In the 1980s, the MCM protein was first isolated from budding yeast with mutants that were defective in the initiation of DNA replication (Maine et al., 1984). Today, we know ten MCM proteins (MCM1-MCM10); but the MCM protein family includes only eight of them (MCM2-MCM9). The MCM1 and MCM10 proteins do not have MCM domain and therefore do not belong to the family (Rubisz et al., 2021). They improved the stability of genome replication by inhibiting the rate of DNA replication in eukaryotic cells (Wang et al., 2020), and small changes in physiological MCM levels will increase genomic instability and tend to increase the incidence of tumor formation (Sedlackova et al., 2020). Subsequently, many studies have confirmed that MCM family members play an important role in the occurrence and development of gastric, kidney, lung, brain, breast and ovarian cancers. However, MCM has been poorly

studied in HNSC. Therefore, a comprehensive study of MCM family members in HNSC will help improve the diagnosis of HNSC and provide new prognostic and therapeutic targets for this refractory disease.

In this study, we analyzed the expression of MCM family in HNSC using the ONCOMINE database and Gene Expression Profiling Interactive Analysis (GEPIA) Then, the expression of MCM family and its correlation with HNSC prognosis were analyzed using Kaplan-Meier plotter database. Finally, 77 cases of head and neck squamous cell (HNSC) and 50 cases of normal tissue samples and relevant clinical data were collected from 2008 to 2012. Immunohistochemistry was used to analyze the expression levels of MCM3, MCM5 and MCM6 in HNSC patients' tissues and the impact of the expression levels on the prognosis of patients, proving that the MCM proteins family is a potential biomarker of HNSC.

Materials and methods

ONCOMINE database analysis

ONCOMINE database (www.oncomine.org) is an open cancer-related database (Ning et al., 2018). We used ONCOMINE to analyze the transcriptional expression of 8 different MCM members in different tumor tissues and their corresponding adjacent normal tissues. Difference in transcriptional expression was compared by students' t test. Cut-off of p-value and fold change were the following: p-value: 0.01, fold change: 2, gene rank: 10%, data type: mRNA.

Table 1. Baseline characteristics of 77 tissue samples of HNSC.

Parameters	Number
Age	
≤60	21
>60	56
Gender	
Male	40
Female	37
Site	
Tongue	29
Gingiva	28
Pharynx	8
Buccal	12
Tumour stage	
I+II	37
III+IV	40
Tumour size	
T1+T2	41
T3+T4	36
Lymph nodes	
N0	66
N1-3	11
Smoking history	
Yes	23
No	54
The history of drinking	
Yes	10
No	67

GEPIA database analysis

GEPIA (http://gepia.cancer-pku.cn/) is an online open analysis site with data from TCGA and GTEx data centers, containing nearly 10,000 tumor and normal tissue data samples (Tang et al., 2017). This database was employed to analyze the expression of 8 different MCM members between different HNSC tissues and normal tissues.

Kaplan-Meier plotter database analysis

Kaplan-Meier plotter (http://kmplot.com/analysis/) including 21 kinds of cancer specimens such as breast cancer, ovarian cancer, lung cancer and stomach cancer, was able to analyze the impact of more than 5000 genes on survival in patients with open database (Nagy et al., 2018). The influence of expressions of MCM mRNA on prognosis in HNSC patients was analyzed by Kaplan-Meier plotter database. HNSC patients were split into high and low expression groups based on median value of mRNA expression and validated by Kaplan-Meier survival curves, with the hazard ratio (HR) with 95% confidence intervals (CI) and log-rank p-value.

Patients and tissue samples

We collected 136 HNSC tissues and 73 normal tissues treated in the First Affiliated Hospital of School of Medicine, Shihezi University, Xinjiang Province from 2008 to 2012. All patients received no treatment before surgery and had no other medical history. Finally, 77 HNSC tissues with complete clinicopathological and follow-up data were collected (Table 1). 50 normal tissues were obtained from the adjacent normal tissues. The clinical staging of selected cases in this study was based on the American Joint Committee on Cancer (AJCC 8th). The research group conducted follow-up once a year, and the follow-up data of this study was completed by July 30, 2020.Three cancerous and one non-cancerous tissue cores (1 mm in diameter) were cut longitudinally from deep layers of cancerous tissue in each paraffin block and mounted on new paraffin blocks with fine steel needles to generate tissue microarrays. This study was approved by the Ethics Committee of the First Affiliated Hospital of Shihezi University (No. 2021-050-01), and the informed consent of each patient was obtained.

Immunohistochemistry

Two-step EnVision method was used for immunohistochemical experiments in this study. First, tissue chips were cut into tissue sections and adsorbed on the slide. The fat was then removed, rehydrated

before heat-induced antigen extraction by EDTA buffer, followed by blocking the endogenous peroxidase activity with 3% hydrogen peroxide. Nonspecific antigen staining was blocked with 3% BSA, then the primary antibodies MCM3(1:8000, 15597-1-AP, Proteintech, Wuhan, China), MCM5(1:8000, 13347-2-AP, Proteintech, Wuhan, China) and MCM6(1:8000, 13347-2-AP, Proteintech, Wuhan, China) were incubated overnight at 4°C. On the next day, the tablets were redyed and sealed with hematoxylin after coloring with DAB solution for 1 minute. The results of immunohistochemical staining were evaluated by 2 pathologists with double-blind method, and the immune response score (IRS) was calculated as the percentage of positive cells multiplied with the intensity of cell staining (Table 3). According to IRS values, the results were divided into low expression group (≤6 points) and high expression group (>6 points). Section repetition was performed when tissue chip staining was atypical.

Statistical analysis

SPSS.23 software was used for statistical analysis of all experimental data in this study. The Kappa test was used to assess the pathologists' degree of agreement in the immunohistochemical analysis. The chi-square test and Fisher's exact test were used to examine the correlation between the expression levels of MCM3, MCM5 and MCM6 and the clinicopathological characteristics of patients with HNSC. For survival analyses, Kaplan-Meier survival curves were constructed, and the differences were tested by the log-rank test. Overall survival (OS) was defined as the time between the date of surgery and the date of death or the date of last contact. Multiple Cox proportional hazards regression (backward, stepwise) was performed to identify the independent factors with a significant impact on patients' survival, and the hazard ratios (HRs) and 95% confidence intervals of the prognostic factors were calculated. *P*-value was calculated based on double-tailed statistical analysis, $P<0.05$ was considered statistically significant.

Results

Aberrant expression of MCM family in HNSC

To explore the distinct prognostic and potential therapeutic value of different MCM members in HNSC patients, mRNA expression of MCMs was analyzed by ONCOMINE database and GEPIA database. The mRNA expression of 8 MCM family members in 20 types of cancers was first measured and compared to normal tissues by ONCOMINE database. We found that the expressions of MCM2-MCM8 mRNA were significantly higher in HNSC tissues for multiple datasets (Fig. 1, Table 2).

In HNSC 6 dataset, MCM2 high-expression was found in HNSC tissues compared with normal tissues, as follows, Cromer et al. (2004) observed (p=4.72E-07) in

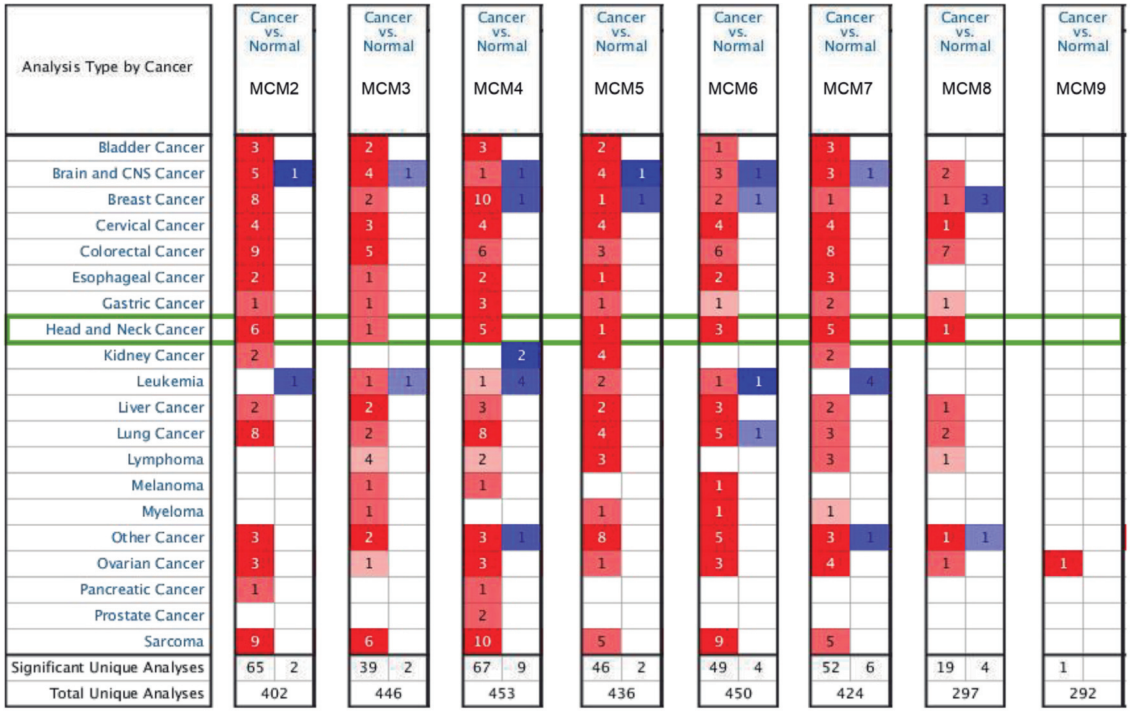

Fig. 1. Transcriptional expression of MCMs in 20 types of cancer diseases (ONCOMINE database). Difference of transcriptional expression was compared by students' t test. Cut-off of *P* value and fold change were as following: *P* value: 0.01, fold change: 2, gene rank: 10%, data type: mRNA.

HNSC, Toruner et al. (2004) observed (p=3.64E-05) in Oral Cavity Squamous Cell Carcinoma (OCSCC), Pyeon et al. (2007) observed (*P*=1.24E-05) in Oral Cavity Carcinoma (OCC), Ye et al. (2008) observed (*P*=7.26E-05) in OCC, Ginos et al. (2004) observed (p=1.43E-08) in HNSC, Sengupta et al. (2008) observed (*P*=1.43E-08) in Nasopharyngeal Carcinoma (NC). In HNSC 1 dataset, MCM3 high-expression was found in HNSC tissues compared with normal tissues by Frierson et al. (2002) who observed (*P*=1.42E-06) in Salivary Gland Adenoid Cystic Carcinoma (SGAC). In HNSC 5 dataset, MCM4 high-expression was found in HNSC tissues compared with normal tissues by Ye Huiet et al. who observed (*P*=1.59E-06) in Tongue Squamous Cell Carcinoma (TSCC), Sengupta et al. (2008) observed (*P*=2.43E-08) in NC, Estilo et al. (2009) observed (*P*=2.43E-08) in TSCC, Pyeon et al. (2007) observed (p=6.61E-06) in Tongue Carcinoma, Pyeon et al. (2007) observed (*P*=9.80E-05) in Floor of the Mouth Carcinoma (FMC). In HNSC 1 dataset, MCM5 high-expression was found in HNSC tissues compared with normal tissues by Pyeon et al. (2007) who observed (*P*=2.41E-05) in Tonsillar Carcinoma. In HNSC 3 dataset, MCM6 high-expression was found in HNSC tissues compared with normal tissues by Pyeon et al. (2007) who observed (*P*=2.88E-06) in OCC, Ginos et al. (2004) observed (*P*=1.96E-13) in HNSC, Frierson et al. (2002) observed (*P*=5.91E-06) in SGAC. In HNSC 5 dataset, MCM7 high-expression was found in HNSC tissues compared with normal tissues by Pyeon et al. (2007) who observed (*P*=6.02E-

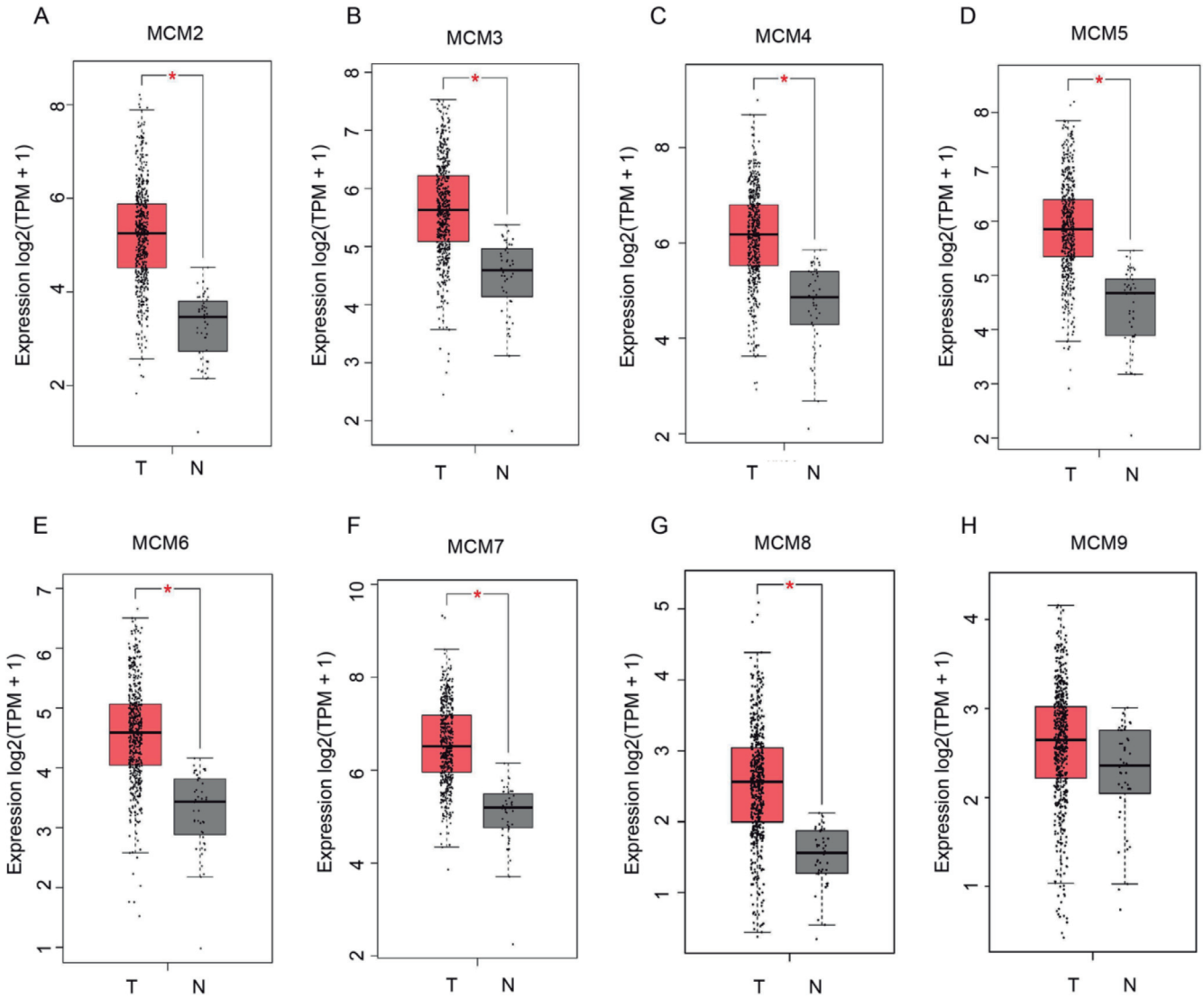

Fig. 2. mRNA expression of distinct MCM family members in HNSC tissues and adjacent normal tissues (GEPIA). mRNA expression of MCM3-MCM8 were found to be overexpressed in HNSC tissues compared to normal tissues **(A-G)**. **P*<0.05.

07) in OCC. Pyeon et al. (2007) observed (*P*=2.47E-05) in FMC, Frierson et al. (2002) observed (*P*=1.85E-05) in Salivary SGAC, Cromer et al. (2004) observed (*P*=1.85E-05) in HNSC, Sengupta et al. (2008) observed (*P*=2.54E-07) in NC. In HNSC 1 dataset, MCM8 high-expression was found in HNSC tissues compared with normal tissues by Sengupta et al. (2008) who observed (*P*=4.82E-08) in NC (Table 2).

Table 2. Significant changes of MCM expression in transcription level between HNSC and normal tissues (ONCOMINE).

	Types of HNSC vs. Normal	Fold change	p-Value	t-test	References
MCM2	Head and Neck Squamous Cell	2.945	4.72E-07	11.981	Cromeret al., 2004
	Oral Cavity Squamous Cell Carcinoma	2.109	3.64E-05	5.116	Toruner al., 2004
	Oral Cavity Carcinoma	2.676	1.24E-05	6.897	Pyeon et al., 2007
	Tongue Squamous Cell Carcinoma	2.116	7.26E-05	4.318	Ye et al.,2008
	Head and Neck Squamous Cell	6.523	1.43E-08	2.347	Ginos et al., 2004
	Nasopharyngeal Carcinoma	2.618	6.63E-06	6.465	Sengupta al., 2006
MCM3	Salivary Gland Adenoid Cystic	43.987	1.42E-06	7.231	Frierson et al., 2002
MCM4	Tongue Squamous Cell Carcinoma	2.120	1.59E-06	5.518	Ye et al.,2008
	Nasopharyngeal Carcinoma	2.249	2.43E-08	7.014	Sengupta al., 2008
	Tongue Squamous Cell Carcinoma	2.303	3.34E-07	5.875	Estilo et al., 2009
	Tongue Carcinoma	2.384	6.61E-06	5.487	Pyeon et al., 2007
	Floor of the Mouth Carcinoma	2.915	9.80E-05	9.211	Pyeon et al., 2007
MCM5	Tonsillar Carcinoma	3.129	2.41E-05	6.068	Pyeon et al., 2007
MCM6	Oral Cavity Carcinoma	2.024	2.88E-06	5.817	Pyeon et al., 2007
	Head and Neck Squamous Cell Carcinoma	2.054	1.96E-13	10.169	Ginos et al., 2004
	Salivary Gland Adenoid Cystic Carcinoma	2.958	5.91E-06	5.814	Frierson et al., 2002
MCM7	Oral Cavity Carcinoma	3.466	6.02E-07	6.624	Pyeon et al., 2007
	Floor of the Mouth Carcinoma	5.942	2.47E-05	6.764	Pyeon et al., 2007
	Salivary Gland Adenoid Cystic Carcinoma	241.460	3.68E-08	9.681	Frierson et al., 2002
	Head and Neck Squamous Cell Carcinoma	4.051	1.85E-05	9.120	Cromer et al., 2004
	Nasopharyngeal Carcinoma	2.229	2.54E-07	7.751	Sengupta et al., 2008
MCM8	Nasopharyngeal Carcinoma	3.390	4.82E-08	7.277	Sengupta et al., 2008

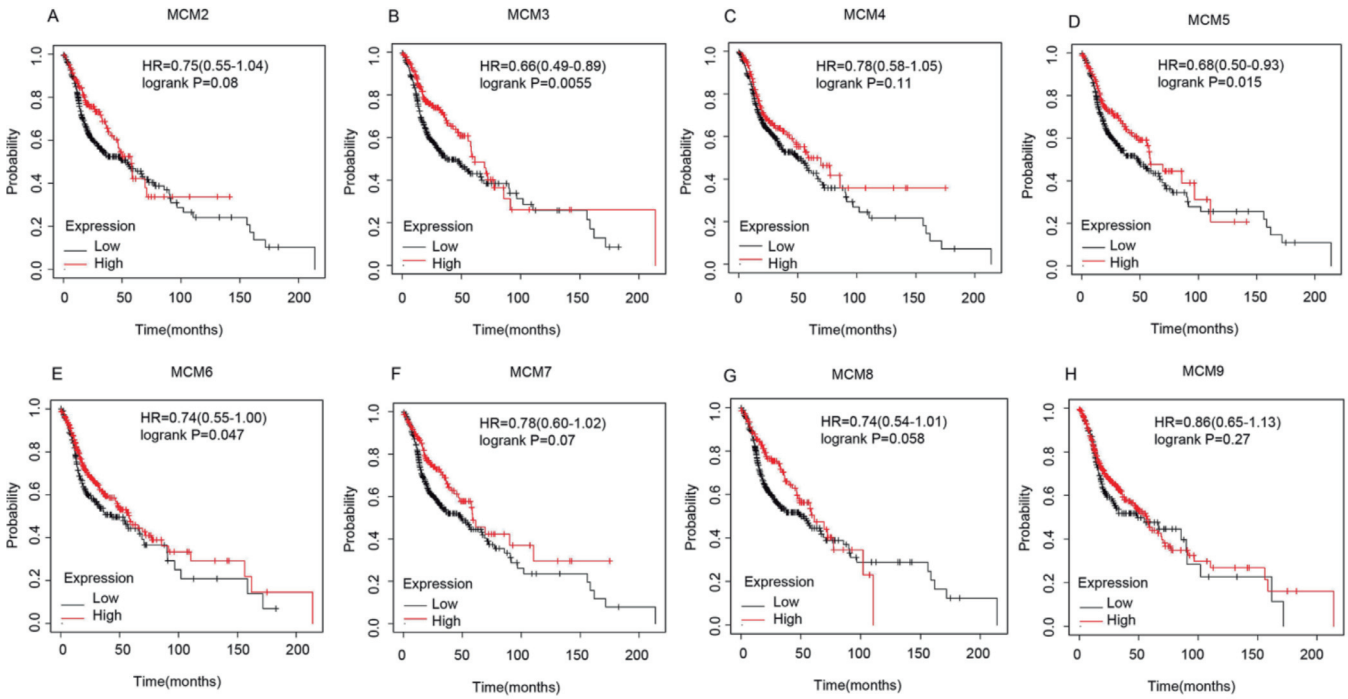

Fig. 3. Prognostic value of mRNA expression of distinct MCM family members in HNSC patients (Kaplan-Meier Plotter). High expressions of MCM3, MCM5, MCM6 in mRNA level were associated with better OS in HNSC patients **(B, D, E)**.

GEPIA database was used to further detect the mRNA expression levels of 8 MCM family members in HNSC. The expression of MCM2-MCM8 mRNA in HNSC was significantly higher than that in normal tissues ($P<0.05$), while there was no significant difference in MCM9 expression ($P>0.05$). In summary, our results suggested that the transcriptional expression of MCM2-MCM8 was overexpressed in HNSC patients (Fig. 2).

Table 3. Immunohistochemical score table.

Positive cells (%)		Intensity		IRS
Percentage	Score	Color	Score	Total score
<5%	0	No color	0	0-1
6~25%	1	Yellow	1	2-4
26~50%	2	Tan	2	5-8
51~75%	3	Brown	3	9-12
76~100%	4			

Relationship between MCM family members' expression and patient prognosis in HNSC

Kaplan-Meier Plotter database analyzed the relationship between mRNA expression of MCM family members and the prognosis of HNSC patients. It

Table 4. Positive expression rates of MCM3, MCM5, MCM6 in HNSC tissues and normal tissues.

Protein and pathology type		Number	Positive	Negative	χ^2	*P*
MCM3	HNSC	77	43	34	13.205	<0.0001
	Normal tissues	50	11	39		
MCM5	HNSC	77	39	38	8.666	0.003
	Normal tissues	50	14	36		
MCM6	HNSC	77	42	35	11.573	0.001
	Normal tissues	50	12	38		

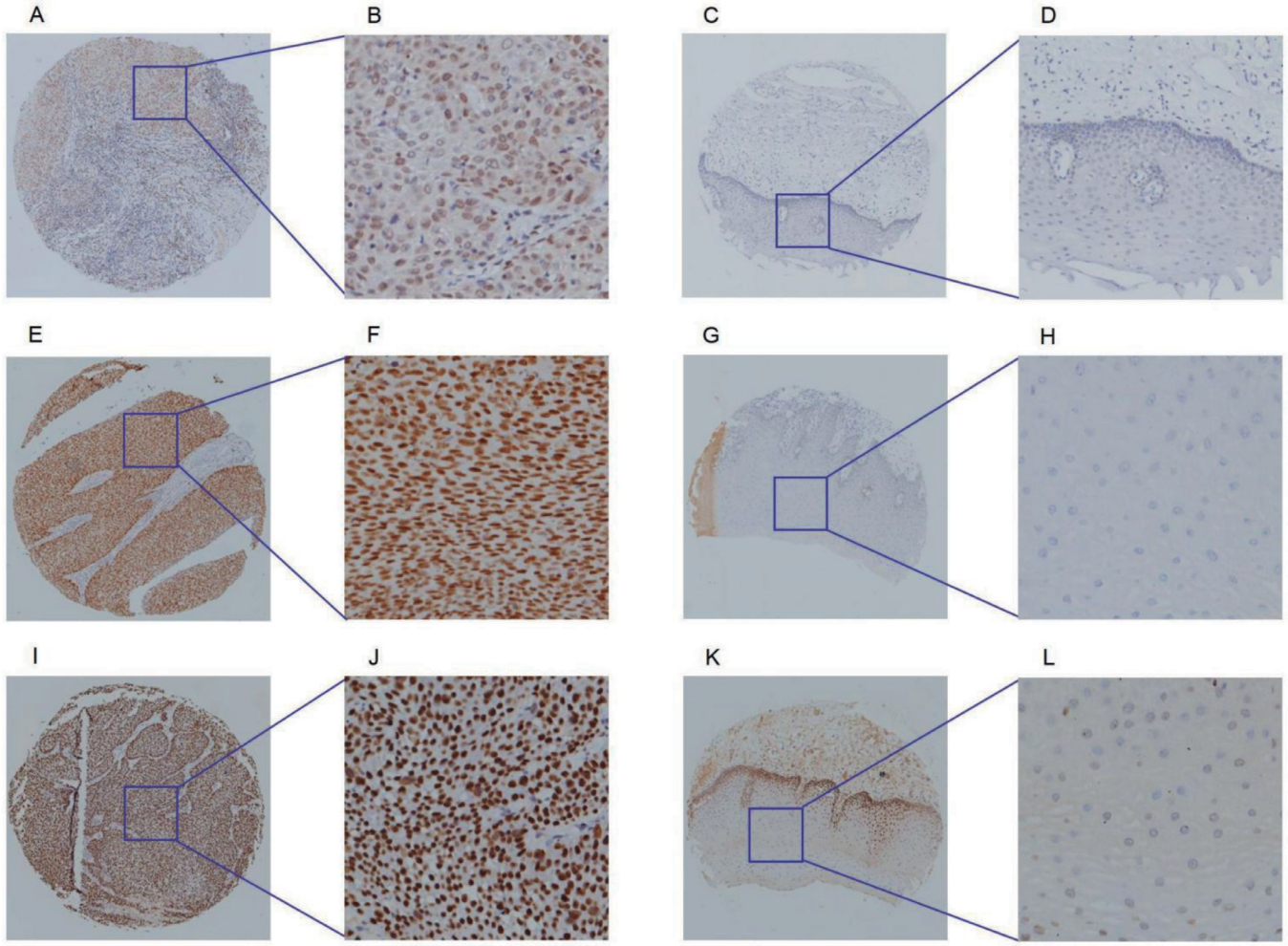

Fig. 4. Immunohistochemical images of MCM3, MCM5, MCM6 in HNSC and normal tissues. MCM3 **(A, B)**, MCM5 **(E, F)** and MCM6 **(I, J)** were overexpressed in HNSC tissues, whereas MCM3 **(C, D)**, MCM5 **(G, H)** and MCM6 **(K, L)** were not expressed in normal tissues. x 100; x 400.

suggested that high expression of MCM3 (HR=0.66, 95% CI: 0.49-0.89, and p=0.0055), MCM5 (HR=0.68, 95% CI: 0.5-0.93, and *P*=0.015) and MCM6 (HR=0.74, 95% CI: 0.55-1, and *P*=0.047) were associated with longer OS of HNSC patients. However, high expression of MCM2 (HR=0.75, 95% CI: 0.55-1.04, and *P*=0.08), MCM4 (HR=0.78, 95% CI: 0.58-1.05, and *P*=0.11), MCM7 (HR=0.78, 95% CI: 0.6-1.02, and *P*=0.07), MCM8 (HR=0.74, 95% CI: 0.54-1.01, and *P*=0.058) and MCM9 (HR=0.86, 95% CI: 0.65-1.13, and *P*=0.27) had no correlation with prognosis of HNSC patients (Fig. 3). These results indicated that mRNA expressions of MCM3, MCM5 and MCM6 were associated with patient prognosis and they may be exploited as useful biomarkers for prediction of HNSC. It is worth noting that MCM8 (*P*=0.058) showed no significant difference, but it was very close to statistical difference, which may be a statistical variation, and needs to be explored in future studies.

Expression rates of MCM3, MCM5 and MCM6 in HNSC tissue and normal tissue

Immunohistochemical results showed that MCM3, MCM5 and MCM6 proteins were mainly distributed in the nucleus of HNSC cells, which were brownish or yellow-brown. There was little staining in normal tissue (Fig. 4).

Kappa-test showed that there was a significant consistency between the two pathologists in the scores of MCM3, MCM5 and MCM6 immunohistochemical groups (*P*<0.0001, Kappa=0.602), (*P*<0.0001, Kappa=0.553) and (*P*<0.0001, Kappa=0.458). Positive expression rate of MCM3, MCM5 and MCM6 in 70 HNSC tissues was 55.84% (43/77), 50.65% (39/77), and 54.55% (42/77), respectively. The positive expression rates of MCM3, MCM5 and MCM6 in 50 normal tissues were only 22% (11/50), 28% (14/50) and 24% (12/50), respectively.This suggested that the expression rates of MCM3, MCM5 and MCM6 in HNSC tissues was significantly higher than in normal tissues (*P*<0.05; Table 4).

Relationship between the expression of MCM3, MCM5, MCM6 and the clinicopathologic characteristics of patients with HNSC.

The expression levels of MCM3 and MCM6 proteins had a significant correlation with the tumor stage and tumor size (*P*<0.05), and the expression of MCM5 protein had a significant correlation with the tumor stage, tumor size and lymph nodes (*P*<0.05) in 70 HNSC tissues. In contrast, no significant associations were observed between MCM3, MCM6 and age, gender, lymph nodes, smoking history or alcohol consumption history (*P*>0.05; Table 5), and no significant associations were observed between MCM5 and age, gender, smoking history or alcohol consumption history (*P*>0.05; Table 5). In brief, the results above suggest that MCM3 and MCM6 were related to tumor stage and tumor size, and MCM5 was related to tumor stage, tumor size and lymph nodes in HNSC patients.

The impact of MCM3, MCM5, MCM6 expression on overall survival (OS) in HNSC

To assess the impact of MCM3, MCM5 and MCM6 expression on prognosis in HNSC patients, Kaplan Meier plotter was used to assess the association between MCM3, MCM5, MCM6 expression and OS. The results suggested that the survival time of patients with high expression of MCM3, MCM5 and MCM6 was statistically different from those with low expression of MCM3, MCM5 and MCM6 (*P*<0.05, Fig. 5). In other words, patients with high expression of MCM3, MCM5

Table 5. Relationships between the expressions of MCM3,MCM5,MCM6 and the clinicopathologic characteristics in HNSC patients.

Parameters		Number	MCM3 expression				MCM5 expression				MCM6 expression			
			Low	High	χ^2	*P*	Low	High	χ^2	*P*	Low	High	χ^2	*P*
Age	≤60	21	9	12	0.020	0.888	9	12	0.487	0.485	7	14	1.711	0.191
	>60	56	25	31			29	27			28	28		
Gender	Male	40	18	22	0.024	0.877	20	20	0.014	0.906	22	18	3.059	0.080
	Female	37	16	21			18	19			13	24		
Tumour stage	I+II	37	22	15	6.765	0.009	27	10	15.901	<0.0001	24	13	10.823	0.001
	III+IV	40	12	28			11	29			11	29		
Tumour size	T1+T2	41	23	18	5.072	0.024	27	14	9.555	0.002	25	16	8.521	0.004
	T3+T4	36	11	25			11	25			10	26		
Lymph nodes	N0	66	29	37	0.009	0.925	37	29	8.322	0.004	32	34	1.711	0.191
	N1-3	11	5	6			1	10			3	8		
Smoking history	Yes	23	12	11	0.239	0.625	11	12	0.020	0.965	13	10	0.159	0.690
	No	54	22	32			27	27			22	32		
History of drinking	Yes	10	6	4	1.170	0.279	5	5	0.030	0.861	6	4	0.981	0.322
	No	67	28	39			33	34			29	38		

and MCM6 had a longer postoperative survival time.

Cox regression analysis of MCM3, MCM5, MCM6 expression on the survival of HNSC patients

Univariate Cox regression analysis showed that the survival time of HNSC patients with high expression of MCM3, MCM5 and MCM6 were significantly longer than with low expression (*P*=0.0007, HR=4.515, 95% CI:2.123-9.601), (*P*=0.007, HR=2.919, 95% CI:1.367-6.232), (*P*=0.006, HR=3.107, 95% CI:1.46-6.612, Table 6). Multivariate Cox proportional risk model suggested that MCM3, MCM5 and MCM6 might act as an independent prognostic factor in HNSC patients *P*=0.001, HR=6.366, 95% CI:2.194-18.467), (*P*=0.002, HR=4.348, 95% CI:1.705-11.089), (*P*=0.007, HR=4.705, 95% CI:1.534-14.432, Table 6).

Discussion

Nowadays, with the continuous development of molecular biology, it has been discovered that cancer is caused by genetic, metabolic, inflammatory and epigenetic factors (Ruiz de la Cruz et al., 2021). MCMs family proteins as an important part of complex which regulates precise DNA replication, is considered to be closely related to the occurrence and development of lung squamous cell carcinoma, prostate cancer, lymphoma, breast cancer and other cancers (Mughal et al., 2019). Although individual MCM member have been demonstrated to play key roles in HNSC, the complete MCMs family in HNSC has not been studied. Therefore, this study aimed to analyze the relationship between expressions of different MCMs family members in HNSC and in patient prognosis.

A large number of studies showed that the expression of MCM3 is significantly increased in lymphoma, colon, lung, stomach, kidney, breast cancer, malignant melanoma, cervical squamous cell carcinoma, lung cancer and other multi-cancers (Lee et al., 2010; Nodin et al., 2012; Ashkavandi et al., 2013; Hua et al., 2014; Mughal et al., 2019; Ma et al., 2021). Increased expression of MCM3 protein was associated with poor prognosis in thyroid tumors (Lee et al., 2010), glioma (Hua et al., 2014), salivary gland tumors (Ashkavandi et al., 2013), melanoma (Nodin et al., 2012) and cervical cancer (Ma et al., 2021). This study found the expression of MCM3 in mRNA and protein levels was significantly

Table 6. Univariate and multivariate survival analysis of MCM3, MCM5, MCM6 expression levels in HNSC patients

Variable		Univariate Analysis			Multivariate Analysis		
		HR	95% CI	*P*	HR	95% CI	*P*
MCM3	High vs Low	4.515	2.123-9.601	0.0007	6.366	2.194-18.467	0.001
MCM5	High vs Low	2.919	1.367-6.232	0.007	4.348	1.705-11.089	0.002
MCM6	High vs Low	3.107	1.46-6.612	0.006	4.705	1.534-14.432	0.007
Age	>60 vs ≤60	1.189	0.53-2.668	0.668			
Gender	Male vs Female	1.419	0.65-3.097	0.351			
Tumour stage	I-II vs III-IV	1.413	0.647-3.082	0.353			
Tumour size	T1+T2 vs T3+T4	1.439	0.638-3.244	0.329			
Lymph nodes	N0 vs N1-3	1.356	0.412-4.46	1.356			
Smoking history	Yes vs No	1.107	0.455-2.692	0.819			
Drinking history	Yes vs No	1.631	0.499-5.33	0.491			

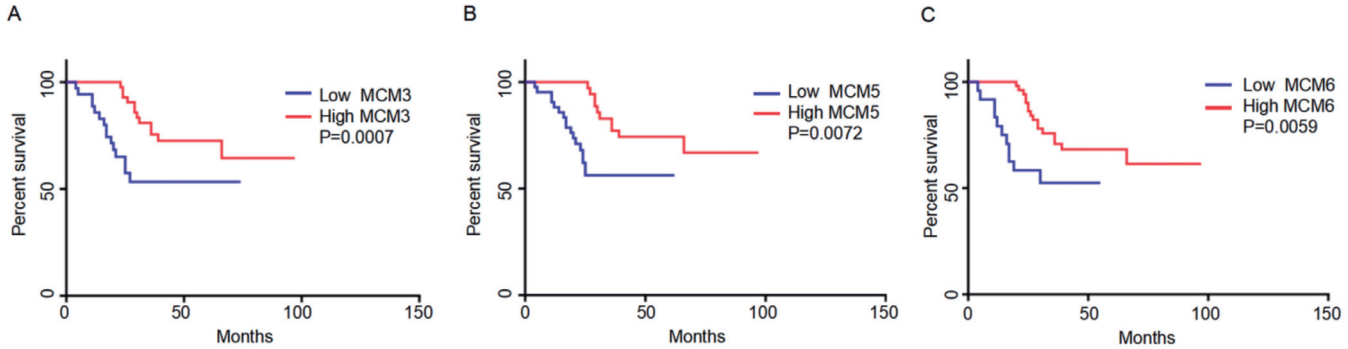

Fig. 5. Survival analysis of MCM3, MCM5, MCM6 expression and the prognosis of HNSC patients. Higher expressions of MCM3,MCM5 and MCM6 were associated with better OS in HNSC patients **(A-C)**.

overexpressed in HNSC tissues, and the expression of MCM3 in protein level was related to patients' tumor stage and tumor size. Further, studies found that MCM3 might promote the occurrence and development of cancer in different ways. In prostate cancer, the expression of MCM3 in normal epithelial cells was significantly lower than in mesenchymal cells, and highly expressed MCM3 may promote the induction of EMT and tumor metastasis (Stewart et al., 2017). In renal cell carcinoma, PLK1 improved the proliferation in cancer cells and inhibited apoptosis by the phosphorylation of MCM3 (Gao et al., 2020). Besides, the high expression of MCM3 in HCC might resist the radiotherapy by activating the NF-κB signaling pathway, but this process might be reversed by the NF-κB inhibitor: JSH-23 (Yang et al., 2019). In addition, we found that highly expressed MCM3 in mRNA and protein levels was also significantly associated with longer OS in HNSC patients, and may be an independent prognostic factor. Our findings suggest that MCM3 is involved in the tumorigenesis of HNSC and it could be a potential prognostic marker.

Studies showed that increased expression of MCM5 was related to tumor size, lymph node metastasis and greater progression of oral squamous cell carcinoma (Yu et al., 2014). High expression of MCM5 was associated with malignancy in gastric adeno carcinoma (Giaginis et al., 2009), bladder cancer (Korkolopoulou et al., 2005), ovarian cancer (Gakiopoulou et al., 2007), and skin cancer (Liu et al., 2007). In our study, significantly higher expression of MCM5 in the level of mRNA and protein were found in HNSC tissues, and the expression of MCM5 in protein level was remarkably correlated with tumor stage, tumor size and lymph nodes. Furthermore, studies found that high expression of MCM5 was related to poor prognosis in patients with liver cancer (Hu et al., 2020), renal cell carcinoma (Gong et al., 2019), gastric adeno carcinoma (Wang et al., 2018) and lung cancer (Zhang et al., 2021). In addition, we found that high expression of MCM5 at the protein level was also significantly associated with better OS in HNSC patients. MCM5 can be used as an independent prognostic factor in HNSC patients, which indicates that MCM5 is involved in the tumorigenesis of HNSC and is a potential prognostic marker. Moreover, MCM5 can eliminate the inhibitory effect of miR-3607 on malignant behavior and epithelial-mesenchymal transformation (EMT) in HCC cells, and then improved the abilities of growth, migration and invasion in HCC cells. A recent study found that MCM5 and HDAC1 work together to promote EMT-dependent malignant progression in lung cancer, whereas, astragaloside IV was able to inhibit the association between MCM5 and HDAC1, thus inhibiting the progression of lung cancer (Zhang et al., 2021).

The expression of MCM6 was significantly increased in cervical cancer, lung cancer, meningioma, chondrosarcoma, craniopharyngioma, mantle cell lymphoma and other cancers (Mughal et al., 2019). This study found the expression of MCM6 in mRNA and protein levels was significantly higher in HNSC tissues, and the expression of MCM6 in protein level was related to tumor stage and tumor size. Besides, high expression of MCM6 was found to be related to poor survival and early recurrence in HCC patients (Liu et al., 2018a,b). Increased expression of MCM6 was correlated with poor prognosis in clear-cell renal cell carcinoma (Jang et al., 2021), adenocarcinoma (Xie et al., 2021), Neuroblastoma (Gu et al., 2021) and gastric cancer patients (Chen et al., 2019). This was consistent with our results, where overexpression of MCM6 in mRNA and protein levels was also significantly associated with longer OS in HNSC patients, and it was an independent prognostic factor in HNSC patients, suggesting that MCM6 is involved in tumorigenesis of HNSC and is a potential prognostic marker. Recent studies have shown that MCM6 can promote the development and metastasis by activating the MEK/ERK signaling pathway in triple-negative breast cancer (Shao et al., 2021). Tumor suppressor CDK5RAP3 can prevent MCM6 from migrating to the nucleus and then inhibiting tumor growth in GC cell lines (Chen et al., 2019). Bleomycin induction significantly inhibit the 53BP1 nuclear chromatin fraction and the focal formation through MCM6 in chemotherapy patients with liver cancer, thus, targeting MCM6 may relieve the chemotherapy response of patients with liver cancer (Chen et al., 2018).

On the whole, our results suggested that in HNSC tissues, mRNA levels of MCM2-MCM8 were overexpressed, and protein levels of MCM3, MCM5 and MCM6 were highly expressed. High expression of MCM3 and MCM6 in protein level was obviously related to tumor stage and tumor size in HNSC patients, and high expression of MCM5 in protein level was significantly associated with tumor stage, tumor size and lymph nodes in HNSC patients. In addition, high expression of MCM3, MCM5 and MCM6 in mRNA and protein levels were obviously related to longer OS in HNSC patients. Univariate Cox regression analysis and Multivariate Cox regression analysis suggested that the survival time of patients with highly expressed MCM3, MCM5 and MCM6 in protein level were significantly longer than that with low expression. In contrast, there was no significant associations with patients' age, gender, tumor stages, tumor size, lymph nodes, smoking history and alcohol consumption history. The findings indicated that MCM3, MCM5 and MCM6 might act as independent prognostic factors in HNSC patients. This means that the survival time of HNSC patients has no significant impact on the clinical characteristics, which may be related to the few cases and the short follow-up time. In addition, the expression of MCM3, MCM5 and MCM6 in HNSC was indeed shown to be a good predictor of the survival time for patients, and our research group intends to further study with later stage HNSC.

Conclusion

Our results showed that overexpression of MCM3, MCM5 and MCM6 in mRNA and protein levels were found in HNSC tissues and they were related to OS in HNSC patients. MCM3, MCM5 and MCM6 were independent prognostic factors for HNSC patients, indicating that MCM3, MCM5 and MCM6 could be prognostic biomarkers for survival of HNSC patients.

Future perspective

HNSC is one of the most frequent neoplasms worldwide, showing aggressive behavior, propensity for lymph-node metastasis, and a poor prognosis. Despite improvements in HNSC treatments, the outcomes of patients with HNSC are still dismal due to the limited knowledge about its molecular pathogenesis, the difficulty in detecting the disease at its early stages, and the lack of effective therapies. Early diagnosis and treatment of HNSC is an important opportunity to improve the prognosis of patients; however, we lack the reliable molecular markers. In this study, biological informatics analysis and immunohistochemical verification methods have proved that MCM3,MCM5 and MCM6 can be used as potential molecular marker for high-risk genotypes in HNSC. However, the significance and molecular mechanism of MCM3, MCM5 and MCM6 in HNSC need to be further verified by a large number of clinical data and long-term follow-up combined with relevant molecular biological experiments. We will further study the significance and mechanism of MCM3, MCM5 and MCM6 in HNSC, and explore the targeted inhibitors to improve the quality of life with HNSC patients.

Availability of data and materials. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests. The authors report no conflicts of interest in this work.

References

Ashkavandi Z.J., Najvani A.D., Tadbir A.A., Pardis S., Ranjbar M.A. and Ashraf M.J. (2013). MCM3 as a novel diagnostic marker in benign and malignant salivary gland tumors. Asian Pac. J. Cancer Prev. 14, 3479-3482.

Bray F., Ferlay J., Soerjomataram I., Siegel R.L., Torre L.A. and Jemal A. (2018). GLOBAL cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. 68, 394-424.

Chen Y., Weng C., Zhang H., Sun J. and Yuan Y. (2018). A direct interaction between P53-binding protein 1 and minichromosome maintenance complex in Hepg2 cells. Cell. Physiol. Biochem. 47, 2350-2359.

Chen Q.Y., Liu L.C., Wang J.B., Xie J.W., Lin J.X., Lu J., Cao L.L., Lin M., Tu R.H., Huang C.M., Li P. and Zheng C.H. (2019). CDK5RAP3 inhibits the translocation of MCM6 to influence the prognosis in gastric cancer. J. Cancer 10, 4488-4498.

Cohen E.E., LaMonte S.J., Erb N.L., Beckman K.L., Sadeghi N., Hutcheson K.A., Stubblefield M.D., Abbott D.M., Fisher P.S., Stein K.D., Lyman G.H. and Pratt-Chapman M.L. (2016). American Cancer Society head and Neck Cancer Survivorship Care Guideline. CA Cancer J. Clin. 66, 203-239.

Cromer A., Carles A., Millon R., Ganguli G., Chalmel F., Lemaire F., Young J., Dembélé D., Thibault C., Muller D., Poch O., Abecassis J. and Wasylyk B. (2004). Identification of genes associated with tumorigenesis and metastatic potential of hypopharyngeal cancer by microarray analysis. Oncogene 23, 2484-2498.

Estilo C.L., O-charoenrat P., Talbot S., Socci N.D., Carlson D.L., Ghossein R., Williams T., Yonekawa Y., Ramanathan Y., O Boyle J., Kraus D.H., Patel S., Shaha A.R., Wong R.J., Huryn J.M., Shah J.P. and Singh B. (2009). Oral tongue cancer gene expression profiling: Identification of novel potential prognosticators by oligonucleotide microarray analysis. BMC Cancer 9, 11.

Frierson H.F. Jr, El-Naggar A.K., Welsh J.B., Sapinoso L.M., Su A.I., Cheng J., Saku T., Moskaluk C.A. and Hampton G.M. (2002). Large scale molecular analysis identifies genes with altered expression in salivary adenoid cystic carcinoma. Am. J. Pathol. 161, 1315-1323.

Gakiopoulou H., Korkolopoulou P., Levidou G., Thymara I., Saetta A., Piperi C., Givalos N., Vassilopoulos I., Ventouri K., Tsenga A., Bamias A., Dimopoulos M.A., Agapitos E. and Patsouris E. (2007). Minichromosome maintenance proteins 2 and 5 in non-benign epithelial ovarian tumours: relationship with cell cycle regulators and prognostic implications. Br. J. Cancer 97, 1124-1134.

Gao Z., Man X., Li Z., Bi J., Liu X., Li Z., Li J., Zhang Z. and Kong C. (2020). PLK1 promotes proliferation and suppresses apoptosis of renal cell carcinoma cells by phosphorylating MCM3. Cancer Gene Ther. 27, 412-423.

Giaginis C., Georgiadou M., Dimakopoulou K., Tsourouflis G., Gatzidou E., Kouraklis G. and Theocharis S. (2009). Clinical significance of MCM-2 and MCM-5 expression in colon cancer: association with clinicopathological parameters and tumor proliferative capacity. Dig. Dis. Sci. 54, 282-291.

Ginos M.A., Page G.P., Michalowicz B.S., Patel K.J., Volker S.E., Pambuccian S.E., Ondrey F.G., Adams G.L. and Gaffney P.M. (2004). Identification of a gene expression signature associated with recurrent disease in squamous cell carcinoma of the head and neck. Cancer Res. 64, 55-63.

Gong B., Ma M., Yang X., Xie W., Luo Y. and Sun T. (2019). MCM5 promotes tumour proliferation and correlates with the progression and prognosis of renal cell carcinoma. Int. Urol. Nephrol. 51, 1517-1526.

Gu Y., Hu X., Liu X., Cheng C., Chen K., Wu Y. and Wu Z. (2021). MCM6 indicates adverse tumor features and poor outcomes and promotes G1/S cell cycle progression in neuroblastoma. BMC Cancer 21, 784.

Hu W.Y., Wei H.Y., Liu L.Y., Li K.M., Wang R.B., Xu X.Q. and Feng R. (2020). miR-3607, a biomarker of hepatocellular carcinoma invasion and aggressiveness: Its relationship with epithelial-mesenchymal transition process. IUBMB life 72, 1686-1697.

Hua C., Zhao G., Li Y. and Bie L. (2014). Minichromosome maintenance (MCM) family as potential diagnostic and prognostic tumor markers for human gliomas. BMC Cancer 14, 526.

Jang N.R., Baek J., Ko Y., Song P.H. and Gu M.J. (2021). High MCM6

expression as a potential prognostic marker in clear-cell renal cell carcinoma. *In Vivo* 35, 299-306.

Korkolopoulou P., Givalos N., Saetta A., Goudopoulou A., Gakiopoulou H., Thymara I., Thomas-Tsagli E. and Patsouris E. (2005). Minichromosome maintenance proteins 2 and 5 expression in muscle-invasive urothelial cancer: a multivariate survival study including proliferation markers and cell cycle regulators. Human. Pathol. 36, 899-907.

Lee Y.S., Ha S.A., Kim H.J., Shin S.M., Kim H.K., Kim S., Kang C.S., Lee K.Y., Hong O.K., Lee S.H., Kwon H.S., Cha B.Y. and Kim J.W. (2010). Minichromosome maintenance protein 3 is a candidate proliferation marker in papillary thyroid carcinoma. Exp. Mol. Pathol. 88, 138-142.

Liu M., Hu Q., Tu M., Wang X., Yang Z., Yang G. and Luo R. (2018a). MCM6 promotes metastasis of hepatocellular carcinoma via MEK/ERK pathway and serves as a novel serum biomarker for early recurrence. J. Exp. Clin. Cancer Res. 37, 10.

Liu Z., Li J., Chen J., Shan Q., Dai H., Xie H., Zhou L., Xu X. and Zheng S. (2018b). MCM family in HCC: MCM6 indicates adverse tumor features and poor outcomes and promotes S/G2 cell cycle progression. BMC Cancer 18, 200.

Liu H., Takeuchi S., Moroi Y., Lin N., Urabe K., Kokuba H., Imafuku S., Dainichi T., Uchi H., Furue M. and Tu Y. (2007). Expression of minichromosome maintenance 5 protein in proliferative and malignant skin diseases. Int. J. Dermatol. 46, 1171-1176.

Ma H., Liu Z., Li H., Guo X., Guo S., Qu P. and Wang Y. (2021). Bioinformatics analysis reveals MCM3 as an important prognostic marker in cervical cancer. Comput. Math. Methods Med. 2021, 8494260.

Maine G.T., Sinha P. and Tye B.K. (1984). Mutants of *S. Cerevisiae* defective in the maintenance of minichromosomes. Genetics 106, 365-385.

Mughal M.J., Mahadevappa R. and Kwok H.F. (2019). DNA replication licensing proteins: Saints and sinners in cancer. Semin. Cancer Biol. 58, 11-21.

Nagy Á., Lánczky, A., Menyhárt O. and Győrffy B. (2018). Author Correction: Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets. Sci. Rep. 8, 11515.

Ning G., Huang Y.L., Zhen L.M., Xu W.X., Jiao Q., Yang F.J., Wu L.N., Zheng Y.Y., Song J., Wang Y.S., Xie C. and Peng L. (2018). Transcriptional expressions of Chromobox 1/2/3/6/8 as independent indicators for survivals in hepatocellular carcinoma patients. Aging (Albany NY) 10, 3450-3473.

Nodin B., Fridberg M., Jonsson L., Bergman J., Uhlén M. and Jirström K. (2012). High MCM3 expression is an independent biomarker of poor prognosis and correlates with reduced RBM3 expression in a prospective cohort of malignant melanoma. Diagn. Pathol. 7, 82.

Pyeon D., Newton M.A., Lambert P.F., den Boon J.A., Sengupta S., Marsit C.J., Woodworth C.D., Connor J.P., Haugen T.H., Smith E.M., Kelsey K.T., Turek L.P. and Ahlquist P. (2007). Fundamental differences in cell cycle deregulation in human papillomavirus-positive and human papillomavirus-negative head/neck and cervical cancers. Cancer Res. 67, 4605-4619.

Rubisz P., Hirnle L., and Kobierzycki C. (2021). The Immunohistochemical expression of MCM-3, -5, and -7 proteins in the uterine fibroids. Curr. Issues Mol. Biol. 43, 802-817.

Ruiz de la Cruz M., de la Cruz Montoya A.H., Rojas Jiménez E.A., Martínez Gregorio H., Díaz Velásquez C.E., Paredes de la Vega J., de la Cruz Hernández-Hernández F. and Vaca Paniagua F. (2021). Cis-acting factors causing secondary epimutations: Impact on the risk for cancer and other diseases. Cancers (Basel) 13, 4807.

Sedlackova H., Rask M.B., Gupta R., Choudhary C., Somyajit K. and Lukas J. (2020). Equilibrium between nascent and parental MCM proteins protects replicating genomes. Nature 587, 297-302.

Sengupta S., den Boon J.A., Chen I.H., Newton M.A., Stanhope S.A., Cheng Y.J., Chen C.J., Hildesheim A., Sugden B. and Ahlquist P. (2008). MicroRNA 29c is down-regulated in nasopharyngeal carcinomas, up-regulating mRNAs encoding extracellular matrix proteins. Proc. Natl. Acad. Sci. USA. 105, 5874-5878.

Shao G., Fan X., Zhang P., Liu X., Huang L. and Ji S. (2021). Methylation-dependent MCM6 repression induced by LINC00472 inhibits triple-negative breast cancer metastasis by disturbing the MEK/ERK signaling pathway. Aging (Albany NY) 13, 4962-4975.

Stewart P.A., Khamis Z.I., Zhau H.E., Duan P., Li Q., Chung L.W.K. and Sang Q.A. (2017). Upregulation of minichromosome maintenance complex component 3 during epithelial-to-mesenchymal transition in human prostate cancer. Oncotarget 8, 39209-39217.

Tang Z., Li C., Kang B., Gao G., Li C. and Zhang Z. (2017). GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res. 45, W98-W102.

Toruner G.A., Ulger C., Alkan M., Galante A.T., Rinaggio J., Wilk R., Tian B., Soteropoulos P., Hameed M.R., Schwalb M.N. and Dermody J.J. (2004). Association between gene expression profile and tumor invasion in oral squamous cell carcinoma. Cancer Genet. Cytogenet. 154, 27-35.

Vigneswaran N., and Williams M.D. (2014). Epidemiologic trends in head and neck cancer and aids in diagnosis. Oral Maxillofac. Surg. Clin. North Am. 26, 123-141.

Wang D., Zhu H., Guo M., Fan X., Hu S., Yan K., Sun J., Wang J., Li M., Xiao H. and Liu Z. (2018). Expression and prognostic value of cell-cycle-associated genes in gastric adenocarcinoma. BMC Gastroenterology 18, 81.

Wang Y., Chen H., Zhang J., Cheng A.S.L., Yu J., To K.F. and Kang W. (2020). MCM family in gastrointestinal cancer and other malignancies: From functional characterization to clinical implication. Biochim. Biophys. Acta. Rev. Cancer 1874, 188415.

Xie J., Peng Y., Chen X., Li Q., Jian B., Wen Z. and Liu S. (2021). LACTB mRNA expression is increased in pancreatic adenocarcinoma and high expression indicates a poor prognosis. PLoS One 16, e0245908.

Yang Q., Xie B., Tang H., Meng W., Jia C., Zhang X., Zhang Y., Zhang J., Li H. and Fu B. (2019). Minichromosome maintenance 3 promotes hepatocellular carcinoma radioresistance by activating the NF-κB pathway. J. Exp. Clin. Cancer Res. 38, 263.

Ye H.,Yu T.,Temam S., Ziober B.L.,Wang J., Schwartz J.L., Mao L., Wong D.T. and Zhou X. (2008). Transcriptomic dissection of tongue squamous cell carcinoma. BMC Genomics 9, 69.

Yu S.Y., Wang Y.P., Chang J.Y., Shen W.R., Chen H.M. and Chiang C.P. (2014). Increased expression of MCM5 is significantly associated with aggressive progression and poor prognosis of oral squamous cell carcinoma. J. Oral Pathol. Med. 43, 344-349.

Zhang F., Liu Y., Yang Y. and Yang K. (2020a). Development and validation of a fourteen- innate immunity-related gene pairs signature for predicting prognosis head and neck squamous cell carcinoma. BMC Cancer 20, 1015.

Zhang X., Shi M., Chen T. and Zhang B. (2020b). Characterization of

the immune cell infiltration landscape in head and neck squamous cell carcinoma to aid immunotherapy. Mol. Ther. Nucleic Acids 22, 298-309.

Zhang L.L., Li Q., Zhong D.S., Zhang W.J., Sun X.J. and Zhu Y. (2021). MCM5 aggravates the HDAC1-mediated malignant progression of lung cancer. Front. Cell Dev. Biol. 9, 669132.

Accepted July 24, 2023