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Original Article

Prognostic significance of MCM6 expression in gastrointestinal stromal tumor

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Abstract: Minichromosome maintenance (MCM) proteins are essential for the initiation of DNA replication and they are prognostic markers in various human cancers. The aim of this study was to investigate the role of the MCM6 protein in gastrointestinal stromal tumor (GIST) and its clinical and prognostic significance. We evaluated MCM6 expression in 211 GIST samples using immunohistochemistry. We used the receiver operating characteristic curve (ROC) to identify optimal cut-off values. High MCM6 expression was associated with tumor size, mitosis, tumor necrosis, presence of recurrence/metastasis, and the National Institute of Health (NIH) and Armed Forces Institute of Pathology (AFIP) malignant risk criteria. Patients with high MCM6 expression had significantly shorter overall survival (OS) and disease-free survival (DFS) than those with low MCM6 expression. Univariate analysis indicated that tumor size, mitosis, AFIP and NIH malignant risk criteria, and high MCM6 expression were significantly associated with poor OS and DFS. High MCM6 expression and high-risk group categorization based on the NIH criteria were independent prognostic factors for OS and DFS. High MCM6 expression is significantly associated with tumor progression and aggressiveness and is an independent factor for shorter survival in GIST patients. MCM6 expression could be a predictive biomarker for tumor aggressiveness as well as a treatment target.

Keywords: Gastrointestinal stromal tumor, MCM6, prognosis

Introduction

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract. They harbor oncogenic mutations in *KIT* (80%-85%) and *PDGFRA* (5%-7%) [1]. The prognosis of GIST varies greatly. Approximately 60% of patients with localized GIST undergo surgery [2]. However, in around 40% of patients, the tumor has already metastasized at the time of diagnosis [3]. Though various prognostic factors of GISTs have been widely studied, tumor size and mitotic count still remain the most widely accepted and clinically valuable prognostic factors. In addition, other parameters such as anatomic location, cellular atypia, tumor necrosis, and perforation are independent prognostic factors of GISTs [4]. Targeted therapies for KIT are widely used in routine practice for advanced GIST and have lead to improvements in survival; however, almost all patients with GIST eventually develop resistance to imatinib. Therefore, identifying potential and objective biomarkers to predict disease aggressiveness and progression is important for formulating effective treatment for GIST.

The minichromosome maintenance (MCM) protein complex is composed of six proteins (MCM2-7). It is a key player in the initiation of DNA synthesis and replication [5, 6]. In addition, the MCM complex contributes to replication elongation, cohesion, condensation, transcription, and recombination of DNA molecules [7]. Each member of the MCM complex plays a distinct role in the regulation of cell behavior [7]. Dysregulation of MCM protein function causes DNA damage, genomic instability, and abnormal cell proliferation [5, 7]. MCM proteins are potential cell proliferation markers in the diagnosis and prognosis of cancer, because they are present in cycling cells and are absent in quiescent cells [6, 8]. Furthermore, MCM pro-

teins are expressed more in malignant cells than in normal cells. MCM6 expression has been evaluated in various human cancers and has been found associated with tumor cell proliferation, invasion, and metastasis [9-15]. However, its prognostic role in mesenchymal neoplasms has not yet been investigated.

The primary goal of this study was to evaluate the expression of MCM6 in GIST and to assess the correlation between its expression and the clinicopathologic variables. The potential of MCM6 as a prognostic biomarker in GIST was assessed. To the best of our knowledge, this is the first study to report MCM6 protein expression and its prognostic significance in GIST.

Materials and methods

Patients and tumour samples

We retrospectively reviewed 248 surgically resected GISTs at Yeungnam University Hospital (1997-2018) and investigated the availability of clinical follow-up data. Inclusion criteria were: histological confirmation of locally advanced and/or metastatic GIST with immunohistochemical (IHC) documentation of c-KIT (CD117) and/or Dog1 expression. After excluding the patients who received preoperative chemotherapy or radiotherapy, had insufficient clinicopathologic data, failed to follow-up, or lacked tissue samples for IHC staining, we included a total of 211 GISTs, comprising samples from the stomach (143 cases), small intestine (64 cases), colon and rectum (each case), and extra-gastrointestinal locations (pelvic cavity and abdominal cavity). The clinicopathologic data pertaining to tumor location, tumor size, mitotic count, tumor cell type, necrosis, mucosal ulceration, and recurrence or metastasis were recorded. The follow-up period ended in October 2020, and the duration of follow-up was calculated from the time of surgical resection to death or the last follow-up (overall survival [OS] range: 1-271 months, mean 82.15 months). Disease-free survival (DFS) was defined as the postoperative interval without recurrence or distant metastasis (DFS range: 0-271 months, mean 74.81 months). The Institutional Human Ethics Review Board (IRB) of Yeungnam University Hospital approved this study (2020-03-034). The waiver for informed consent for this study was obtained from the IRB. The risk of malignant behavior was classified according to the National Institutes of Health (NIH) consensus criteria [16] and Armed Forces Institute of Pathology (AFIP) criteria [17]. NIH classification system utilize two clinical pathological factors, tumor size and mitotic count, allowing recurrence risk to be stratified as very low, low, intermediate, or high [16]. However, GISTs arising from the stomach have generally better prognosis than those arising from the small bowel or rectum, the AFIP criteria incorporate tumor site (stomach, duodenum, ileum/jejunum, and rectum) as well as tumor size and mitotic count and proposed four risk categories as none, very low, low, moderate and high [17].

Construction of tissue microarray block

Whole sections were stained using hematoxylin and eosin to identify the most representative tumor area. Tissue microarrays (TMAs) were constructed using paraffin-embedded donor blocks of the GIST tissues. At least two to five 2-mm cores were taken from the most representative tumor area of each sample and mounted into the recipient paraffin block using a Tissue-Tek® Quick-Ray™. Breast invasive carcinoma, thyroid papillary carcinoma, normal gastric mucosa, palatine tonsil, or uterine leiomyoma samples were arrayed in TMA blocks and used as control tissues.

Immunohistochemistry and interpretation

IHC analysis for assessing MCM6 expression was performed using an automated Benchmark platform (Ventana Medical Systems, Tucson, AZ, USA). Antigen retrieval was performed using Cell Conditioning Solution (mild cc1; Ventana Medical Systems, Tucson, AZ, USA). Anti-MCM6 antibodies (ERP17686, rabbit monoclonal, 1: 1000; Abcam, Cambridge, UK) were added to the samples and incubated for 40 min at room temperature. Sections were visualized using the UltraView Universal DAB Detection Kit (Ventana Medical Systems). Samples that were not treated with primary antibodies served as negative controls.

The slides were scanned using the Leica Aperio ScanScope (Leica Biosystems, Vista, CA, USA) system at 20× magnification, and images were captured using a Leica ImageScope. Manual counting of MCM6-expressing cells was per-

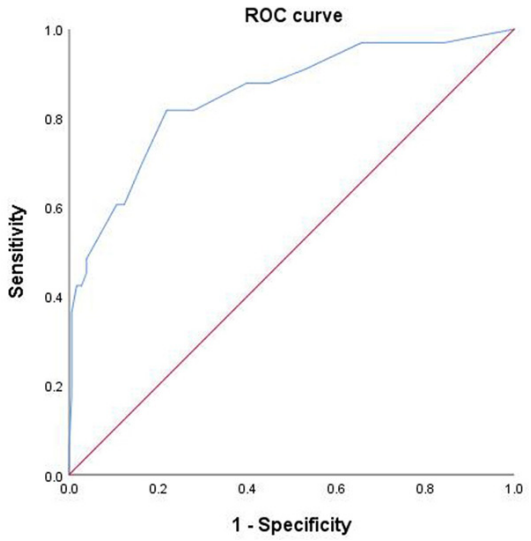

Figure 1. The global predictive accuracy was 0.851% (95% CI: 0.774-0.929, *P*<0.001).

formed on a computer monitor using the scanned images.

We selected two to three hotspots (with a minimum of 500 tumor cells) for each case. All tumor nuclei and MCM6-positive tumor cells were counted from the images, to yield a continuous score from 0 to 100.

Statistical analysis

To identify the optimal cut-off value with the highest accuracy to predict outcome, receiver operating characteristic (ROC) curve analysis was performed. Clinicopathologic data were compared between the GISTs with low and high expression of MCM6, using Fisher's exact test and Chi-square test. Survival rates were calculated using the Kaplan-Meier method. The association between survival rates and various clinicopathologic factors was evaluated using the log-rank test. The Cox proportional hazards regression model was used to evaluate the significance of the prognostic factors. Variables showing significant results in the univariate analysis were further analyzed using multivariate Cox proportional hazards regression analysis. Hazard ratios (HRs) and associated 95% confidence intervals (CIs) were calculated for each variable. All comparisons were performed using IBM SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Differences were considered significant at *P*<0.05.

Results

Clinicopathologic characteristics

A total of 113 men and 98 women with a mean age of 57.6 years (median: 58.0 years, range: 22-88 years) were included in this study. The mean tumor size was 4.92 cm (range: 1-20 cm).

MCM6 expression and its clinicopathologic significance

The cut-off value for MCM6 positivity with the highest accuracy was 15% (range: 0%-95%, mean: 15.8%). The global predictive accuracy (area under the receiver operating characteristic curve) was 0.851% (95% CI: 0.774-0.929, *P*<0.001) (**Figure 1**). Therefore, samples with tumor cells that showed moderate to strong nuclear staining with or without cytoplasmic reaction in more than 15% of the total tumor cells were defined as having high MCM6 expression. Among the 211 GISTs, 65 (30.8%) exhibited high MCM6 expression (**Figure 2**). The percentage of MCM6 expression increased significantly with the increase in tumor size. High MCM6 expression was associated with mitosis (>5/50 high-power fields [HPF]), tumor necrosis, recurrence or metastasis, and NIH and AFIP malignant risk criteria (**Table 1**).

Patients with high MCM6 expression showed significantly shorter OS (252.647 vs. 114.599 months) and DFS (258.126 vs. 104.419 months) than those with low MCM6 expression (both, *P*<0.001) (**Figure 3A**, **3B**). Patients with GIST in the small intestine had a significantly increased risk with respect to OS (HR: 2.517, 95% CI: 1.244-5.493, *P*=0.010) and DFS (HR: 2.941, 95% CI: 1.447-5.978, *P*=0.003) than those with GIST in the stomach. However, this could not be established for extra-GIST or colorectal GIST, because the number of samples was small. In a univariate analysis, tumor size, mitosis, AFIP and NIH malignant risk criteria, and high MCM6 expression were significantly associated with poor OS and DFS. Tumor necrosis was associated with poor OS, but not with DFS. With respect to NIH risk criteria, the high-risk group of GIST had poor OS (HR: 57.183, 95% CI: 7.764-421.147) and DFS (HR: 69.003, 95% CI: 9.307-511.602) than the low-risk group (*P*<0.001). With respect to the AFIP risk criteria, the high-risk group of GIST had poor OS (HR: 53.749, 95% CI: 7.258-398.056)

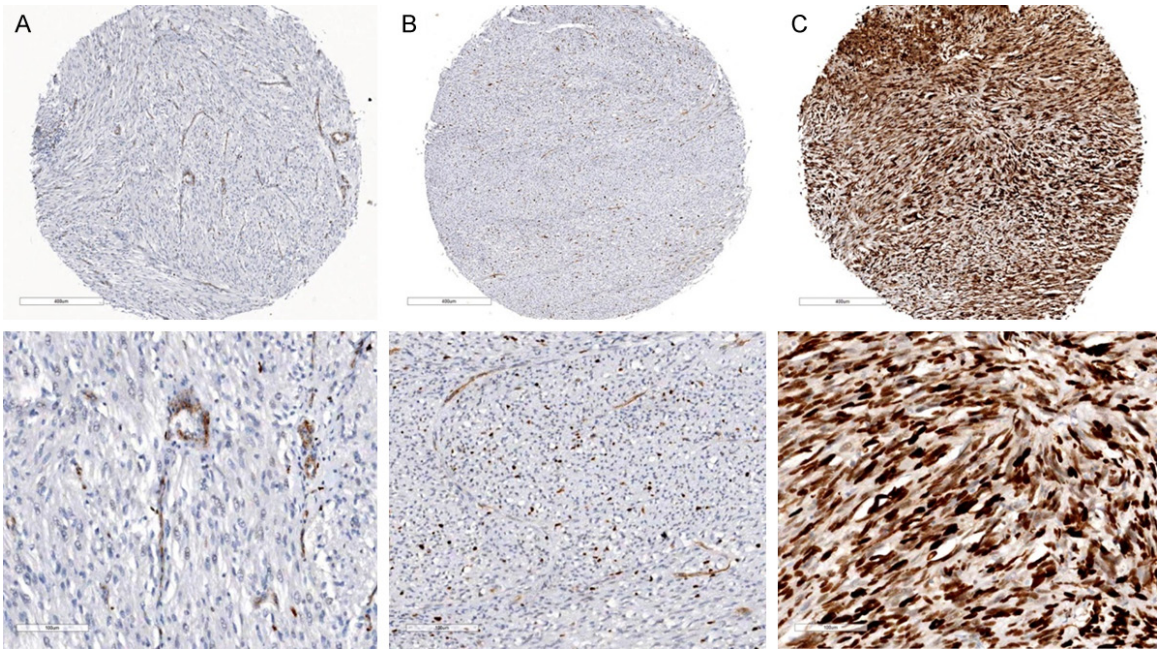

Figure 2. Immunohistochemical staining for MCM6 expression. A. No MCM6 expression in gastrointestinal stromal tumor cells is observed, but inflammatory cells and endothelial cells are positive. B. Low MCM6 expression. C. High MCM6 expression.

and DFS (HR: 61.222, 95% CI: 8.140-460.463) than the low-risk group (P<0.001). Patients with high MCM6 expression had poor OS (HR: 16.676, 95% CI: 6.350-43.795) and DFS (HR: 15.190, 95% CI: 6.201-37.210) than those with low MCM6 expression (P<0.001). In the high-risk group, patients with high MCM6 expression had a shorter OS and DFS than those with low MCM6 expression (P<0.001) (**Figure 4**). Multivariate analysis showed that high MCM6 expression was an independent prognostic factor for OS (P<0.001, HR: 7.318, 95% CI: 2.571-20.828) and DFS (P<0.001, HR: 8.405, 95% CI: 3.193-22.123). The "high-risk" categorization according to the NIH criteria, was an independent prognostic factor for OS (P<0.001, HR: 28.387, 95% CI: 3.695-218.064) and DFS (P=0.004, HR: 49.302, 95% CI: 3.587-675.782) (**Tables 2**, **3**).

Discussion

Patients may have different clinical outcomes despite similar clinicopathological characteristics. Therefore, it is important to explore the clinical relevance of novel biomarkers and possible therapeutic targets. The aggressiveness of GISTs is categorized based on the combination of tumor size, mitotic count, tumor location, and tumor rupture [18]. Cell cycle dysregulation induces a transition from low-risk to high-risk GIST. This is associated with inactivating mutations of the p16, p52, or RB1 tumor suppressor genes. Therefore, these mutations could be useful biomarkers for estimating patient prognosis or for identifying high-risk patients [19, 20]. GISTs with different genetic alterations have different clinicopathological features [21].

DNA replication is essential for cell division during the growth and repair of damaged tissues. MCM proteins are required for DNA replication and are targets of S-phase checkpoints [5, 6]. The increase or inhibition of MCM protein expression results in genomic instability, which could lead to carcinogenesis [22]. Upregulation of MCM6 has been evaluated in and been shown to have prognostic value for various human carcinomas, such as endometrial adenocarcinoma, non-small lung cancer, hepatocellular carcinoma, breast cancer, pancreatic adenocarcinoma, meningioma, and colorectal cancer [7-12, 14]. However, studies on MCM proteins, including MCM6, in soft tissue tumors are limited. MCM6 expression is associated with a higher histologic grade in chondrosarcoma [23]. We found only one study on MCM

Table 1. Correlation between clinicopathologic factors and MCM6 expression in gastrointestinal stromal tumor

Variable	MCM6 expression n (%)		*P*-value
	Low	High	
Sex			0.894
Man	61 (54.0)	52 (46.0)	
Woman	52 (53.1)	46 (46.9)	
Tumor size (cm)			<0.001
≤2	23 (85.2)	4 (14.8)	
>2≤5	87 (75.7)	28 (24.3)	
>5≤10	29 (54.7)	24 (45.3)	
>10	7 (43.8)	9 (56.3)	
Mitosis			<0.001
≤5	112 (85.5)	19 (14.5)	
>5	34 (42.5)	46 (57.5)	
Mucosal invasion			0.078
Present	16 (55.2)	13 (44.8)	
Absent	130 (71.4)	52 (28.6)	
Necrosis			<0.001
Present	41 (63.1)	24 (36.9)	
Absent	133 (91.1)	13 (8.9)	
Cell type			0.912
Spindle	130 (68.8)	59 (31.2)	
Epithelioid	6 (85.7)	1 (14.3)	
Mixed	10 (66.7)	5 (33.3)	
Recurrent/metastasis			<0.001
Absent	139 (75.1)	46 (24.9)	
Present	7 (26.9)	19 (73.1)	
AFIP			<0.001
Low	69 (84.1)	13 (15.9)	
Intermediate	38 (86.4)	6 (13.6)	
High	39 (45.9)	46 (54.1)	
NIH			<0.001
Low	91 (86.7)	14 (13.3)	
Intermediate	18 (60.0)	12 (40.0)	
High	37 (48.7)	39 (51.3)	

Values are presented as number (%).

expression in GIST [24]. MCM2 expression was associated with tumor size, mitotic activity, and NIH risk criteria. MCM2 expression was not an independent prognostic factor; however, patients with MCM2 expression had a shorter disease-specific survival than those without MCM2 expression [24].

Our results pertaining to the prognostic factors of GIST were consistent with those of previous studies. Larger tumor size (>5 cm), high mitotic count (>5/50 HPF), metastasis, and AFIP and NIH risk criteria were associated with poor survival. High MCM6 expression was significantly associated with tumor size, mitosis, tumor necrosis, presence of recurrence/metastasis, and high-risk categorization according to NIH and AFIP risk criteria. This indicates that MCM6 may play an oncogenic role in GIST progression. Up to 20% of patients show overt metastasis upon diagnosis. Therefore, predicting the risk of recurrence is very important for adjuvant treatment of localized tumors [2]. In this study, four patients (1.9%) had metastasis at the time of diagnosis, and three of them (75%) had high MCM6 expression (more than 30% positivity). Among patients with recurrence/metastasis, patients with high MCM6 expression showed poor OS (120.952 vs. 79.289 months) and DFS (39.286 vs. 26.016 months) than those with low MCM6 expression. However, this difference was not significant (P=0.437 and P=0.240). We speculate that high MCM6 expression may promote recurrence/metastasis and poor survival.

Considering the NIH risk criteria, patients with high-risk GIST had worse OS and DFS than those with low or intermediate GIST. In addition, patients with high-risk GIST showing high MCM6 expression had poor OS (93.982 months; HR: 4.827, 95% CI: 1.833-12.710) and DFS (68.679 months; HR: 5.271, 95% CI: 2.151-12.918) than those with low MCM6 expression (OS and DFS of 207.258 months and 220.564 months, respectively). Both the NIH risk criteria and high MCM6 expression were independent prognostic factors; however, high MCM6 expression was a statistically more reliable predictor of prognosis. MCM proteins are markers for cancer screening, surveillance, and prognosis [25, 26]. MCM6 immunohistochemistry is easy to perform in routine practice and MCM6 is a highly reliable prognostic marker; therefore, MCM6 expression may be an efficient indicator of tumor aggressiveness and could be used to identify high-risk groups of NIH and AFIP criteria when diagnosing needle biopsy or small biopsy samples.

In conclusion, this is the first study to assess the prognostic significance of MCM6 expression in GIST. High MCM6 expression is corre-

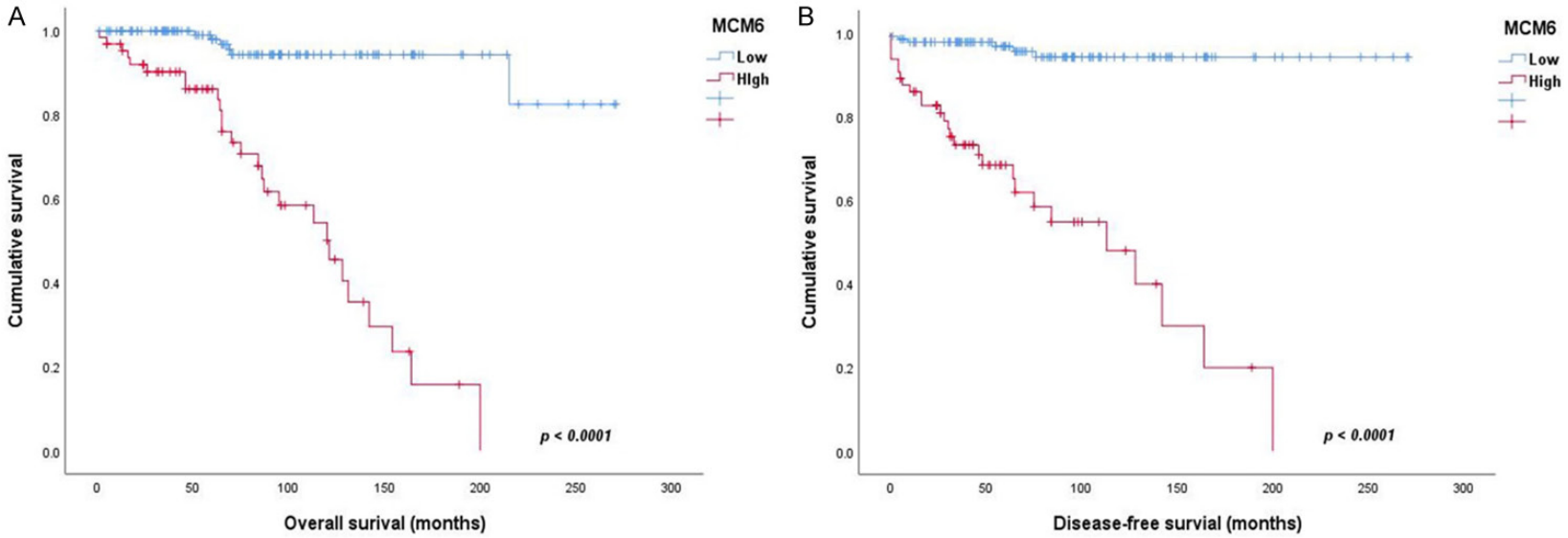

Figure 3. Kaplan-Meier survival curves for the whole patient cohort according to MCM6 protein expression. A. Cumulative overall survival. B. Disease-free survival. High MCM6 expression was significantly associated with worse survival.

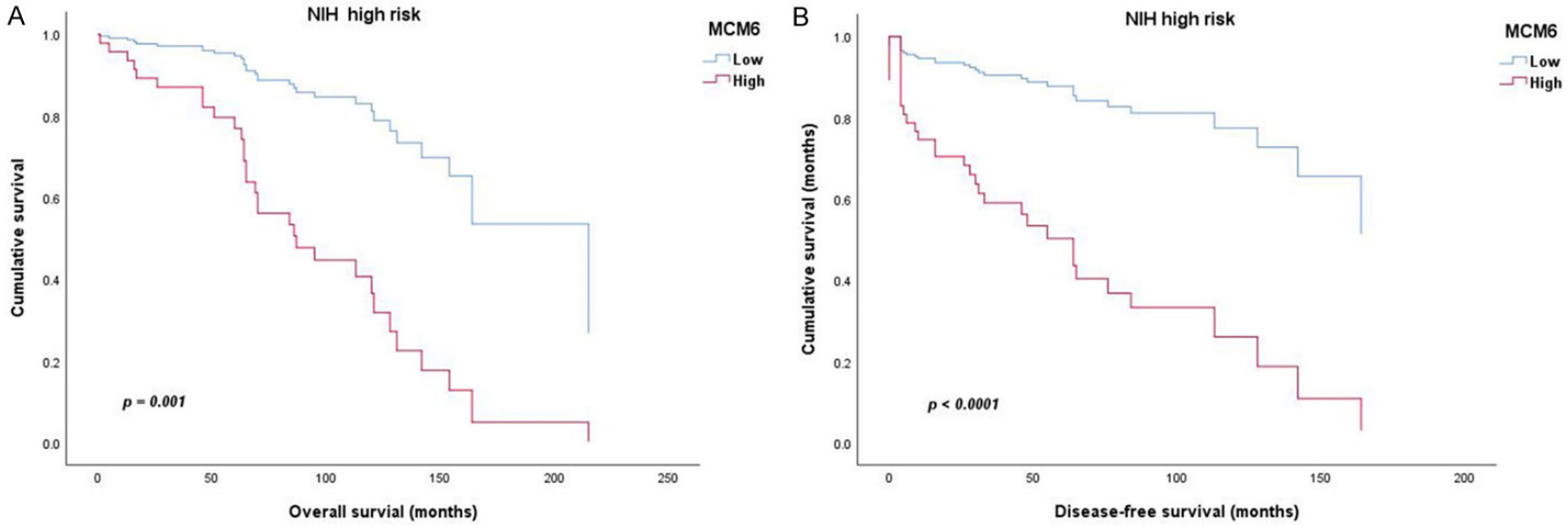

Figure 4. Survival curves according to MCM6 protein expression in patients with high risk for GISTs categorized according to the NIH criteria. Those with high MCM6 expression had significantly worse overall (A) and disease-free (B) survival.

Table 2. Univariate and multivariate analyses of clinicopathologic factors affecting the overall survival of patients with gastrointestinal stromal tumor

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Size (cm)				
≤2		<0.001		0.355
>2≤5	2.637 (0.314-22.141)	0.372	1.963 (0.214-17.971)	0.550
>5≤10	12.917 (1.706-97.790)	0.013	1.978 (0.237-16.511)	0.529
>10	43.200 (5.255-355.111)	<0.001	4.193 (0.486-36.137)	0.192
Mitosis	19.045 (6.631-54.698)	<0.001	0.916 (0.201-4.184)	0.910
Necrosis	9.240 (4.446-19.205)	<0.001	0.743 (0.303-1.819)	0.515
AFIP risk				
Low		<0.001		0.857
Intermediate	2.711 (0.169-43.449)	0.481	0.724 (0.003-201.709)	0.911
High	53.749 (7.258-398.056)	<0.001	1.724 (0.003-892.912)	0.864
NIH risk				
Low		<0.001		<0.001
Intermediate	4.861 (0.304-77.801)	0.264	3.913 (0.242-63.198)	0.336
High	57.183 (7.764-421.147)	<0.000	28.387 (3.695-218.064)	<0.001
MCM6 expression	16.676 (6.350-43.795)	<0.001	5.034 (1.655-15.312)	<0.001

Table 3. Univariate and multivariate analyses of clinicopathologic factors affecting disease-free survival of patients with gastrointestinal stromal tumor

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Size (cm)				
≤2		<0.001		0.355
>2≤5	1.992 (0.237-16.747)	0.526	1.963 (0.214-17.971)	0.550
>5≤10	12.523 (1.647-95.237)	0.015	1.978 (0.237-16.511)	0.529
>10	38.527 (4.693-316.264)	<0.001	4.193 (0.486-36.137)	0.192
Mitosis	21.393 (7.317-62.545)	<0.001	0.916 (0.201-4.184)	0.910
AFIP risk				
Low		<0.001		0.901
Intermediate	2.511 (0.156-40.296)		0.955 (0.001-1392.416)	0.990
High	51.222 (8.140-460.463)		1.901 (0.001-4447.184)	0.871
NIH risk				
Low		<0.001		<0.001
Intermediate	4.344 (0.271-69.554)	0.299	4.622 (0.224-95.386)	0.322
High	69.003 (9.307-511.602)	<0.001	49.302 (3.597-675.782)	0.004
MCM6 expression	15.190 (6.201-37.210)	<0.001	8.405 (3.193-22.123)	<0.001

lated with aggressive clinicopathologic variables and poor survival in GIST. MCM6 is a highly reliable prognostic marker and can be effectively used as a biomarker for the evalua-

tion of prognosis. It may also serve as a therapeutic target for GISTs. However, large-scale studies are needed to verify the cut-off values for the expression of MCM6 and validate these results.

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Disclosure of conflict of interest

None.

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