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MCM2 expression levels predict diagnosis and prognosis in gastric cardiac cancer

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Summary. Background: Gastric Cardiac Cancer (GCC) has high incidence and poor prognosis requiring early screening of high-risk populations. Minichromosome maintenance (MCM) proteins are used as diagnostic-biomarkers in many cancers but not validated for GCC. We evaluate MCM protein 2 (MCM2), comparing it with the validated markers Ki67 and PCNA. Methods: GCC and corresponding cardiac precancerous samples were immunostained with Ki67, MCM2 and PCNA antibodies. Results: 90% of dysplasia samples expressed MCM2, whereas Ki67 and PCNA were expressed in 67% and 80% respectively. The sensitivity and negative predictive values of MCM2 were also superior at 90% and 87%, respectively. Ki67 and PCNA expression was correlated with MCM2, but their expressions seldom reached surface layers, whereas MCM2 manifested mostly in easily accessible superficial layers. Labeling indices (LI) of Ki67 and PCNA were also lower. Significant associations between LI (MCM2), LI (PCNA), and TNM-stages, lymph node metastases and GCC grade were found ($P<0.05$). Increased protein expressions were associated with reduced overall and disease-free survival ($P<0.05$). Although Ki67 and PCNA were significant prognostic factors, there was no significant improvement in multivariate statistical analyses, in contrast to LI (MCM2) findings. Conclusions: MCM2 is a sensitive, specific and efficient biomarker of GCC having potential use in clinic.

Key words: Minichromosome maintenance protein 2 (MCM2), Gastric cardiac carcinoma, Biomarker, Immunohistochemical staining

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Introduction

Gastric Cardiac Cancer has one of the highest incidence rates in China and mortality related to cardiac cancer (GCC) in particular (as opposed to distal gastric cancer) and accounts for almost 12% of all deaths (Jemal et al., 2003; Parkin et al., 2005). GCC has epidemiologic and pathologic features that are very similar to esophageal cancer. In high risk districts of China (e.g. Linzhou and Chaoshan region) and also in some other countries, such as South Africa, GCC accounts for about 40% of malignant tumors, which are characterized by a progressive dysphagia (Li et al., 1989). An increase in morbidity from GCC during recent years is evident and the annual increase rates in morbidity have accelerated to 4~10% in some western countries (Zheng et al., 1993), which is in contrast to the descending trends observed in distal gastric tumor epidemiology.

The molecular mechanisms underlying carcinogenesis in GCC is still elusive, and only recently molecular markers have been included as diagnostic tools for cancer evaluation. An uncontrolled abnormal proliferation of cells is a hallmark of malignancy and its degree is closely correlated to prognosis. Dysplasia is an altered state, a deviation from the normal which conventionally describes an intermediate phase between normal epithelial growth and neoplasia (DeVita et al., 1993). Thus, dysplasia alone could imply an increased risk of cancer and the need for increased surveillance (Wei et al., 2005). Accompanying the precancerous transition is a number of changes in cell morphology, nuclear size and shape. Traditional methods of identifying dysplastic cell populations, such as assessment of cytomorphology and identification of increased number of mitoses by Hematoxylin and Eosin (HE) staining alone, have often been found to be variable among different observer (Mulder et al., 1992),

mainly due to the subjective nature of the methods and this decreases their predictive ability. Although a number of staining indices have attempted to standardize the identification of dysplastic cell populations and abnormal cell behavior, most have frequently been proven to be limited in their ability to ascertain patient prognoses. Traditional biomarkers such as Ki67 and PCNA have had a wide application in the assessment of cell proliferation as reported in several previous studies (Sasano et al., 1992; Barrett et al., 1997), but these indicators sometimes fail to predict relapses accurately, even in patients in early-stages of cancer. This is partly because of the heterogeneous behavior of the markers within the current GCC staging and also because of Ki67's ambiguity and PCNA's non-specificity (Hall et al., 1990; Mangham et al., 1994; Barrett et al., 1997). Consequently, new biomarkers for cancers are required, to better diagnose various forms of dysplasia in an effort to prevent cancer and also to improve survival.

There are a number of studies that have sought to assess the potential prognostic value of molecular markers in predicting the course of GCC, but hitherto still no molecular method to improve risk assessment is in routine clinical use. Thus, as classification of tumor stage remains the most accurate prognostic factor for survival in patients with GCC, an early detection and surveillance of the precancerous lesion stage (dysplasia) and early lesion of GCC become essential to improve survival rates. Gastroscope and forceps biopsy are the most prevailing screening techniques employed in detection of precancerous lesions and early GCC in high risk populations. As samples from these methods are limited to the surface layer cells, pathology diagnoses by HE staining are prone to false negative classifications and also to subjective variability (Reid et al., 1988). Patients with early-stage disease are treated primarily by surgical resection with curative intent and advances have been made in surgical techniques and also in post-operative caring, but due to the highly aggressive nature of GCC, the prognosis of symptomatic GCC is poor and mortality rate continues to rise - even in patients who accept complete resection, the prognosis is dismal, with 30%-70% of patients dying due to recurrence. Thus, there is a lack of a sensitive and specific biomarker for effective detection, prevention and intervention and efforts to develop prognosis markers that help to improve survival in patients with completely resected GCC are essential.

Minichromosome maintenance protein (MCM) is a family of six highly conserved and highly homologous proteins (MCM2-7). The MCM2-7 polypeptides form a functional hexameric complex (Prokhorova and Blow, 2000) that comprises an important part of the 'pre-replicative complex' of replication proteins at replication origins during the G1 phase. The protein then irreversibly dissociates to ensure that DNA synthesis is initiated only once during each cell cycle (Stillman, 1996; Tye, 1999; Tachibana et al., 2005). Further, the expression of MCM is confined to replicating cells in both normal and abnormal human tissue (Freeman et al., 1999), and not evident in quiescent, differentiated and senescent cells, and all of the six MCM proteins show similar and comparable expressions in a range of tissue sections (Stoeber et al., 2001). These features qualify MCM as promising candidates for biomarkers of cancer.

Here, we examined MCM2 protein expressions using immunohistochemistry (IHC) staining of precancerous and cancerous tissue from 180 GCC operative specimens and compared the MCM2 protein levels with clinical outcome to test its role as a potential prognostic and predictive biomarker in the management of GCC. For comparison purposes, we also illuminated the expressions of Ki67 and PCNA, two proteins known to indicate cellular proliferation in cancer.

Materials and methods

Patients and surgical samples

Tumor samples and histological reports from 180 patients with GCC TNM stages I-IV, who had undergone surgical resection at the Tumor Hospital of Shantou University Medical College between October 2000 and October 2007, were collected. The tumor samples were carefully viewed for location of the Z-line (level of transition of pale squamous epithelium of the esophagus to the velvety-red gastric epithelium). The distal esophagus was defined as the area 5 cm above the Z-line, and the gastric cardia was defined as the most proximal 3 cm of the stomach. According to the WHO/IARC guidelines, gastric cardiac cancer was located below the gastro-esophageal junction and was centered within 3 cm from the junction. Location and size of tumors was recorded. The specimens were used for diagnostic evaluation and examined for IHC expressions according to ethical protocols established at Shantou University and all patients signed an informed consent. In addition, 47 corresponding cardiac precancerous samples [including 17 hyperplasia (proliferation of glandular epithelium cells) and 30 dysplasia samples] were taken from the mucosa adjacent to the GCC tumor. Further, 10 normal cardiac specimens were obtained from autopsy cases of non-cardiac disease from the department of Pathology at Shantou university medical college. All the study subjects signed informed-consent documents and the study was approved by the ethical review committees of the Shantou University.

Immunohistochemistry

Immunohistochemical staining was performed using Envision Labeled Peroxidase System (ELPS) method. The formalin-fixed, dehydrated and paraffin-embedded tissues were sectioned into 5 mm slices. These were deparaffinized with xylene (3x5 mins) and rehydrated through graded ethanol (90, 75, and 50%) for 2 mins at each concentration. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide–methanol for 12

mins, and rinsed with distilled water. The sections were placed in EDTA solution (0.01M, PH 6.7) and heated in a microwave oven for 2 and 5 mins for Ki67 and MCM2, respectively, for antigenicity retrieval. Citrate buffer solution (0.01M, PH 6.0) was used for 10 mins for PCNA retrieval. Following antigen retrieval, slides were cooled to room temperature for 20 mins, and then washed twice with 0.01M phosphate-buffered saline (PBS). Primary antibodies for Ki67 [Mouse anti-human (ZM-0166)], PCNA [Mouse anti-human (PC10), both from Zhongshan Golden-Bridge Biotechnology Co.; working solution] and MCM2 [Mouse anti-human monoclonal (CRCT5.1) from Neomarkers, Lexington, KY; 1:100 dilution] were applied and incubated at 4°C overnight. Antigen–antibody complexes were rinsed with PBS solution to remove unbound antibodies and were processed using a DAKO Envision kit (Dako North America, Inc., Carpinteria, CA, USA) for 30 mins at room temperature. The slides were incubated with corresponding secondary antibodies for 30 mins, and were stained first with diaminobenzidine tetrahydrochloride (DAB), and then counterstained with Mayer's hematoxylin, differentiated in 1% acid alcohol. After dehydration in graded alcohol and clearance in xylene, the slides were mounted with Canada balsam. Incubation of specimens with PBS and/or pre-immune serum, instead of the primary antibodies, was used as negative control. Tonsil tissue sections were used as positive controls for Ki67 and MCM2, and mammary cancer tissue sections for PCNA.

Digital images were acquired with Leica Imagine-microscope (Leica, Wetzlar, Germany) using Leica software with standard settings in image brightness and contrast mode, and Leica Qwin standard Y2.8 software was used to analyze the results. Images from Ki67, MCM2 and PCNA protein stained sections were captured under high power magnification (x400). For each of the proteins, 3 different fields were randomly chosen and captured separately and independently of each other. The labeling indices (LI, %) for each sample were calculated using the formula: number of positively stained nuclei (in the 3 scans) / the total number of tumor cell nuclei counted (in the 3 scans) x 100%. The expressions were considered positive (+) if >5% of the cells had a dark brown stained nucleus and the results defined negative (-) when ≤5% of the cells stained. Further, a LI value between 25-50% was denoted "++"; and >50% as "+++". In accordance with the pathological gold standard, each LI value in dysplasia diagnosis was expressed as true positive (TP); true negative (TN); false positive (FP); or false negative (FN). TP was defined as positive IHC and HE findings in dysplasia samples; FP as positive IHC findings in non-dysplasia samples by HE staining; FN as negative IHC findings in dysplasia samples by HE staining; and TN as negative IHC findings in non-dysplasia samples by HE staining. Sensitivity defined as TP/ (TP + FN); specificity as TN/ (TN + FP); accuracy as (TP + TN)/ (TP + FN + TN + FP); positive predictive value as TP/ (TP + FP); and negative predictive value as TN/ (TN + FN) were also determined.

Analysis of prognostic factors for survival

To evaluate the 5-year survival rate of patients with GCC, the medical records and tissue samples from surgical cases were carefully examined and all cases having both, the paraffinized sections and complete medical records were reviewed. The Borrmann classification for gross morphology and the WHO classification for histopathology were followed (Fenoglio-Preiser et al., 2000). Cancer-stage was determined according to the 5th edition of the International Union Against Cancer TNM classification (Sobin, 1997). We examined the medical records of each patient and the Ki67, MCM2 and PCNA proteins to identify the factors that could influence the prognosis. The Medical records provided information, such as date of birth, gender, chemotherapy, TNM stage, lymph node metastases, time point when the local recurrence or metastatic disease was first identified and its cancer stage, date of the last medical examination, time and cause of death. We contacted the families of every patient individually to confirm that survival or date of death matched those in records. The first identified date of recurrent or metastatic disease was used in the analysis of disease-free survival (DFS) and this was used in preference to the actual survival since it is recognized to be a more sensitive indicator of an aggressive disease. Prognostic factors for DFS and overall survival (OS) were investigated using the following eleven clinically pathological variables; the host factors were: age (at diagnosis) and gender; the surgical factors were: presence/absence of lymph node metastasis, surgery with adjuvant chemotherapy or chemotherapy-naïve alone; the seven tumor factors were: pathological tumor size, TNM Clinical Staging, T staging, N staging, LI of Ki67, LI of MCM2 and LI of PCNA.

Statistical analysis

All data are presented as the percentage of patients or as the mean with standard deviation of parameters. Categorical variables were compared by chi-square test as appropriate, and continuous variables were compared by the Student's t-test. One-way analysis of variance (ANOVA) was used for LI analysis. The relationship between categorical variables was analyzed by Spearman correlation analysis.

Kaplan-Meier cumulative survival curves were constructed for Ki67, MCM2 and PCNA to evaluate DFS and OS, and differences in survival between the groups were compared by log-rank test. Multivariate analysis of the data was carried out on the survival data. Variables that were shown to significantly influence survival in univariate analysis were included in stepwise multivariate analysis using Cox's regression model to assess the association between each potential prognostic

factor and OS. A value of P <0.05 was considered statistically significant. All data were computerized and analyzed using SPSS 13.0 for Windows statistical computer software.

Results

Demographic features of the patients

A total of 180 GCC patients, 151 men and 29 women with a mean age of 57 years (range, 35-81 years), underwent cardiac resections and 27 of these cases also received adjuvant chemotherapy after the surgery. There were 148 cases of tubular adenocarcinoma (AC); classified as well differentiated (10), moderately differentiated (64) and poorly differentiated (74). The remaining cases included mucoid carcinoma (26), papillary AC (2) and adenosquamous carcinoma (4). All patients were followed up one month post-surgery and then every three months. When entogastric recurrence was suspected, patients were admitted to receive X-ray and endoscope examination. Out of the 180 GCC patients, there is complete follow-up data for 133 cases, of which 126 cases were followed-up for no less than 5 years, a rate of 95%.

IHC expression of Ki67, MCM2 and PCNA in cardiac precancerous mucosa

IHC expression and the labeling indexes of Ki67, MCM2 and PCNA in cardiac precancerous mucosa are presented in Figs. 1, 3. In normal cardiac glandular

Table 1. IHC expression of Ki67, MCM2 and PCNA in cardiac precancerous mucosa and GCC.

	Cases	LI (mean±SD)		
		Ki67	MCM2	PCNA
N	10	0.03±0.02	0.04±0.03	0.10±0.08
H	17	0.09±0.06[1*]	0.15±0.08[4*]	0.19±0.17[7*]
D	30	0.30±0.15[2*]	0.40±0.15[5*]	0.35±0.13[8*]
GCC	180	0.48±0.11[3]	0.60±0.09[6*]	0.53±0.13[9]
Total	237	0.44±0.11	0.55±0.16	0.50±0.13

*: denotes statistically significant; N: Normal; H: Hyperplasia; D: Dysplasia; GCC: gastric cardiac cancer; [1, 4, 7]: H compare with N by LI (Ki67), LI (MCM2) and LI (PCNA) respectively; [2, 5, 8]: D compare with H; [3, 6, 9]: GCC compare with D.

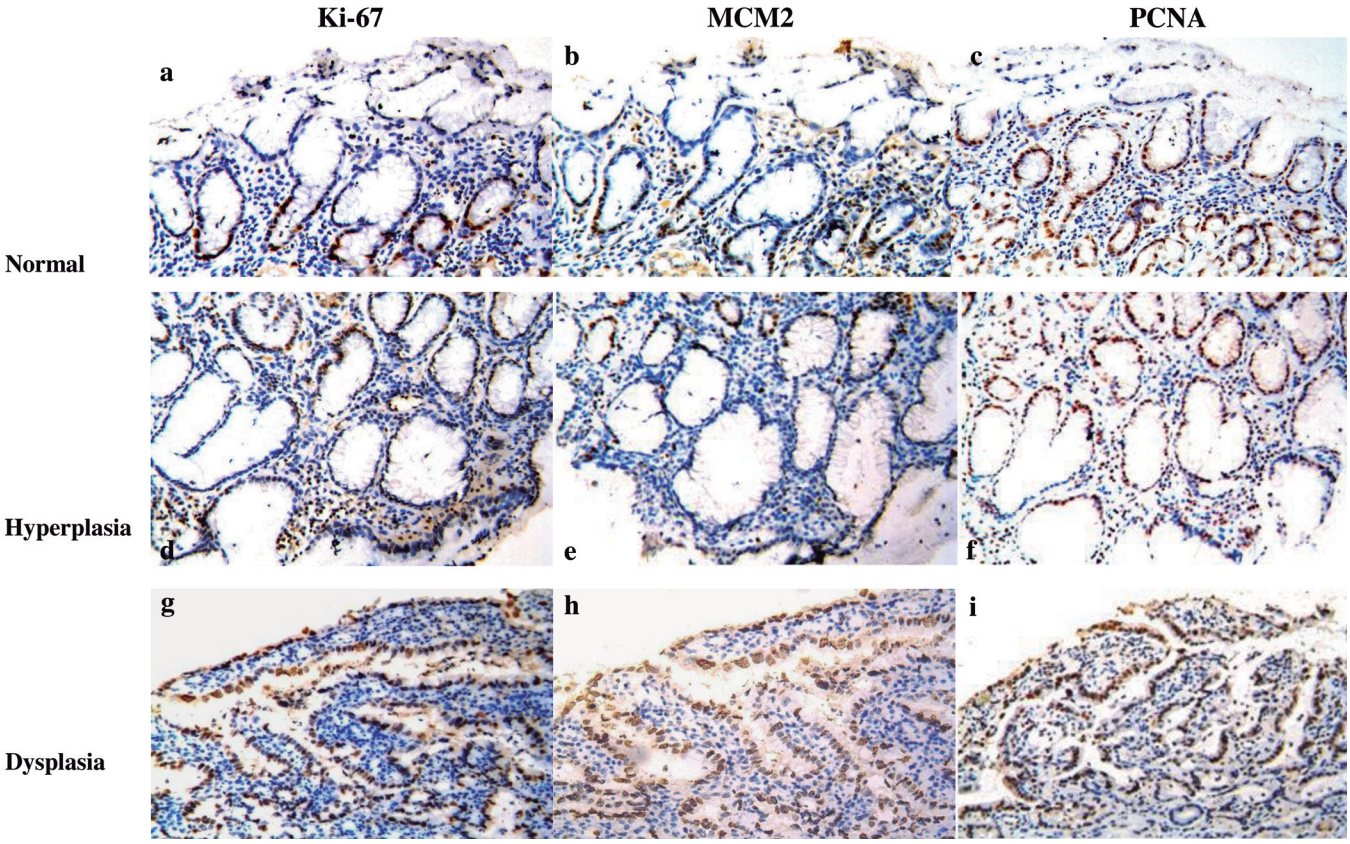

Fig. 1. IHC expression of Ki67, MCM2 and PCNA in cardiac precancerous mucosa from adjacent to GCC tumor. Results of Ki67, MCM2 and PCNA in normal **(a, d, g)**, hyperplasia **(b, e, h)** and dysplasia mucosa **(c, f, i)**. x 200

mucosa, Ki67, MCM2 and PCNA were generally restricted to the nuclei of basal proliferative compartments as shown by the dark brown staining. There is no expression in surface cells of gastric cardiac mucosa or interstitial cells (Fig. 1a-c). In contrast, the areas with proliferation of simple hyperplasia have expression of all three markers extended from the bottom to the middle part (Fig. 1d-f). PCNA-positive stained nuclei are present in the middle layers of normal glandular epithelium (Fig. 1c) and LI of PCNA was found to be higher than Ki67and MCM2 in the normal and hyperplasia cardiac mucosa, but the difference was not statistically significant (P>0.05) (Tables 1, 2). In dysplastic samples taken from mucosa adjacent to tumor Ki67, MCM2 and PCNA were expressed at a higher frequency in the bottom, middle and top layers of the epithelium (Fig. 1g-i). The LIs of Ki67, MCM2 and PCNA showed similar patterns of variation between dysplasia samples, although the overall MCM2 LI values were higher than those for Ki67 and PCNA (Tables 1, 2). However, the expressions of Ki67 and PCNA did not always reach the surface layer in dysplasia samples (Fig. 1g-i), whereas MCM2 was detected in most of the surface layer cells.

Although the expression of the three markers in normal and hyperplasia were low and relatively similar to each other (Table 1), our results show that MCM2 is better than Ki67 and PCNA in revealing dysplasia in adjacent precancerous mucosal cells in GCC. Out of the 30 dysplasia samples, a majority (27 cases; 90%)

Table 2. Comparison of LI for Ki67, MCM2 and PCNA by p-values in cardiac precancerous mucosa and GCC.

	Cases	LI (MCM2) -LI (Ki67)	LI (MCM2) -LI (PCNA)	LI (Ki67) -LI (PCNA)
N	10	0.905	0.112	0.074
H	17	0.153	0.704	0.13
D	30	0.002*	0.012*	0.481
GCC	180	0.000*	0.000*	0.358

*: denotes statistically significant; N: Normal; H: Hyperplasia; D: Dysplasia; GCC: gastric cardiac cancer.

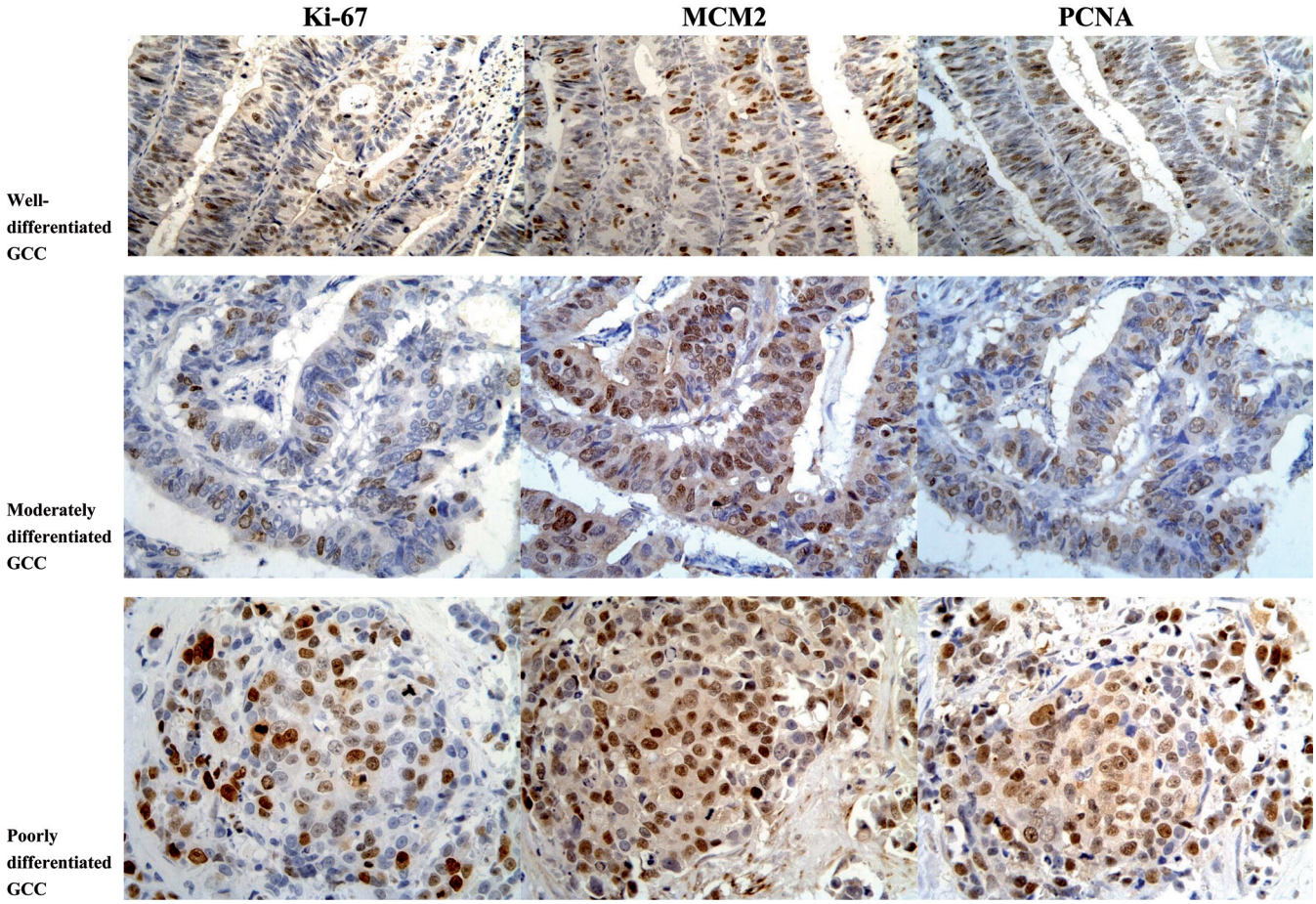

Fig. 2. IHC expression of Ki67, MCM2 and PCNA in different grades of GCC. x 400

expressed MCM2, and this was higher than for Ki67 (20 cases; 66%) and PCNA (24 cases; 80%) (Table 3). Also, there were more MCM2 positive nuclei in these same samples, compared to the corresponding Ki67 and PCNA staining (Fig. 1g-i). Analysis of the expression pattern in the 30 cardiac precancerous mucosa adjacent to GCC suggested that the sensitivity, specificity, accuracy, positive predictive value and negative predictive value of MCM2 in diagnoses of dysplasia were high; 90.0%, 63.0%, 77.2%, 73.0%, 87.0%, respectively, and were consistently higher than those obtained for Ki67 and PCNA (Table 4).

In GCC, the positive rate of Ki67, MCM2 and PCNA was 100% and it was mainly expressed in the basal cells of the cancer nests in well differentiated GCC, but diffusely expressed in moderately and poorly differentiated GCC (Figs. 2,3). There was very widespread expression of these markers, with an overall LI of 48%, 60%, and 53% for Ki67, MCM2 and PCNA respectively. The highest LI values were observed in the poorly differentiated GCCs. In particular, there was strong expression of MCM2 in the surface layer of carcinoma in all cases. The LI of MCM2 protein was higher in GCC compared with the Ki67 and PCNA expressions (P=0.0001) (Table 5). More interestingly, the number of Ki67 positive stained nuclei was always lower than that of MCM2 or PCNA in almost all GCC samples. Also, PCNA positively stained nuclei were present at near the center of the cancer nest in poorly differentiated GCC (Fig. 2). The LI of MCM2 was lowest in well-differentiated cancer and highest in poorly differentiated cancer, and the differences for well vs. moderate and moderate vs. poor were both statistically significant. The LI of PCNA in moderately differentiated cancer was different from the poorly differentiated cancer, and this difference was also statistically significant. The LI of Ki67 in the various grades of GCC were not significantly different.

Table 3. The IHC expression results for Ki67, MCM2 and PCNA in the cardiac precancerous mucosa.

	Ki67		MCM2		PCNA	
	-	+	-	+	-	+
N	6	4 (40%)	7	3 (30%)	4	6 (60%)
H	9	8 (47%)	10	7 (41%)	8	9 (53%)
D	10	20 (66%)	3	27 (90%)	6	24 (80%)

N: Normal; H: Hyperplasia; D: Dysplasia.

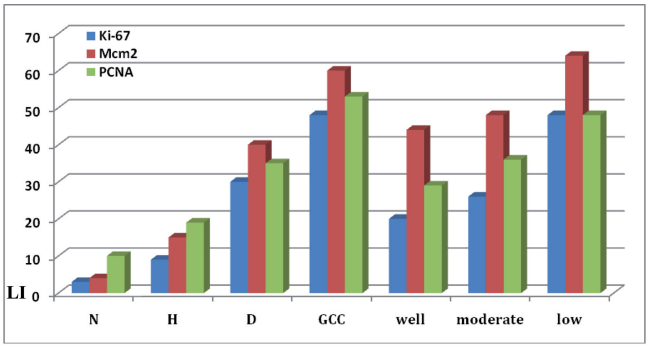

Fig. 3. The LI (Ki67), LI (MCM2) and LI (PCNA) in precancerous mucosa and GCC. N: Normal; H: Hyperplasia; D: Dysplasia; GCC: Gastric Cardiac Cancer; well, moderate, low-different differentiated grades of tubular adenocarcinoma.

Table 4. The power of IHC expression values of Ki67, MCM2 and PCNA in detecting cardiac dysplasia.

	Sensitivity	Specificity	Accuracy	Positive predictive value	Negative predictive value
Ki67	66.7%	55.6%	61.4%	62.5%	60.0%
MCM2	90.0%	63.0%	77.2%	73.0%	87.0%
PCNA	80.0%	44.4%	63.2%	61.5%	66.7%

Table 5. The LI (Ki67), LI (MCM2) and LI (PCNA) in different differentiation grade of tubular adenocarcinoma in GCC (Mean±SD).

Differentiation Grade					ANOVO	
	case	Ki67	MCM2	PCNA	F value	P value
well	8	0.20±0.18	0.44±0.13	0.29±0.18	11.8	<0.001
moderate	48	0.26±0.11[1]	0.48±0.11[3*]	0.36±0.17[5]	15.464	<0.001
poor	51	0.48±0.12[2]	0.64±0.18[4*]	0.48±0.11[6*]	7.381	<0.001
Total	107	0.29±0.12	0.56±0.12	0.41±0.15	71.417	0.001

*: denotes statistically significant; [1, 3, 5]: Moderate compared with well-differentiated GCC; [2, 4, 6]: Poor compared with moderately-differentiated GCC.

As the cells progressed from a normal stage to hyperplasia, to dysplasia and finally to GCC, MCM2 was expressed by an increasing number of cell, in all the epithelial compartments analyzed, but the most striking increase was in the superficial layers. The MCM2 LI for this layer (and, to a lesser extent, the LI of Ki67 and

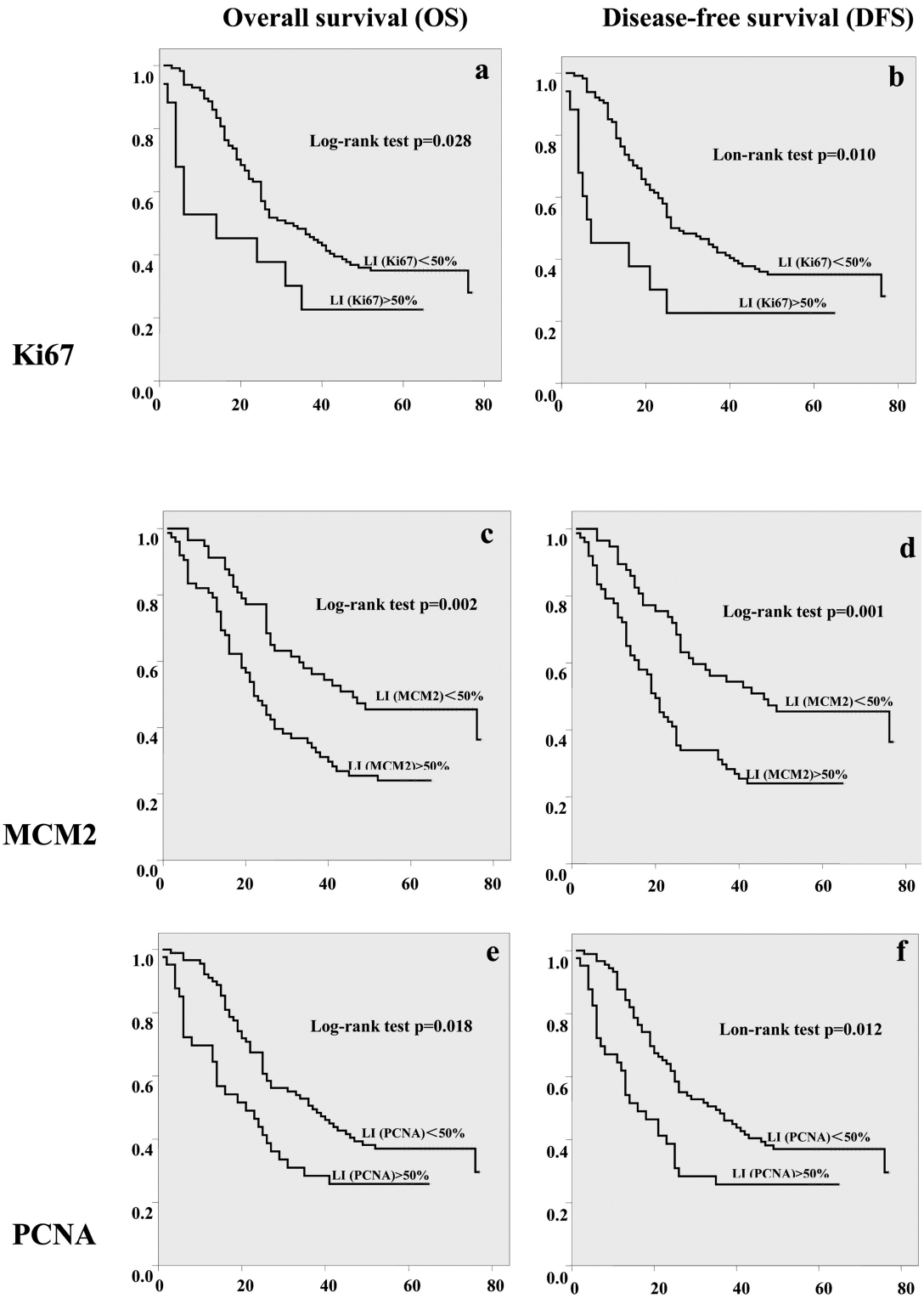

Fig. 4. Overall survival (OS) and Disease-free survival (DFS) according to the pre-operative presence of Ki67, MCM2 and PCNA. **a, b:** OS and DFS according to Ki67 protein expression. **c, d:** OS and DFS according to MCM2 protein expression. **e, f:** OS and DFS according to PCNA protein expression.

Table 6. Ki67, MCM2 and PCNA expressions and clinically pathological features in GCC patients.

Clinically pathological features			Ki67		MCM2		PCNA	
		133 cases	LI (Mean±SD)	p value	LI (Mean±SD)	p value	LI (Mean±SD)	p value
Age (years)	≤53	36	0.30±0.13	0.8	0.50±0.19	0.16	0.38±0.13	0.08
	>53	97	0.30±0.214		0.56±0.24		0.43±0.17	
Gender	Male	110	0.31±0.14	0.7	0.54±0.23	0.61	0.42±0.17	0.67
	Female	23	0.29±0.09		0.57±0.0.21		0.40±0.11	
Tumor size	≤5cm	67	0.30±0.13	0.7	0.54±0.23	0.73	0.42±0.16	0.934
	>5cm	66	0.31±0.14		0.55±0.22		0.42±0.16	
Gross morphology	Borrmann IV	9	0.35±0.17	0.5	0.55±0.29	0.92	0.42±0.19	0.82
	Borrmann III	64	0.30±0.14		0.55±0.23		0.41±0.16	
	Borrmann II	53	0.31±0.13		0.55±0.22		0.43±0.15	
	Others	7	0.24±0.10		0.49±0.30		0.38±0.20	
Pathologic type	Tubular AC	107	0.29±0.12	0.9	0.55±023	0.99	0.41±0.16	0.611
	Mucinous AC	22	0.30±0.11		0.54±0.20		0.41±0.15	
	Papillary AC	2	0.26±0.07		0.50±0.04		0.32±0.03	
	Adenosquamous carcinoma	2	0.27±0.13		0.54±0.48		0.53±0.35	
TNM staging	I	6	0.27±0.10	0.001	0.47±0.27	0.01	0.34±0.18	0.01
	II	28	0.25±0.11		0.43±0.22		0.34±0.14	
	III	63	0.30±0.13		0.56±0.23		0.43±0.17	
	IV	36	0.35±0.16		0.61±0.20		0.46±0.14	
Lymphatic metastasis	Yes	91	0.32±0.15	0.001	0.58±0.22	0.02	0.44±0.16	0.034
	No	42	0.26±0.11		0.49±0.23		0.37±0.16	

AC: adenocarcinoma.

Table 7. The 5-year OS and DFS rates according to the different Ki67, MCM2 and PCNA protein expressions.

Group	cases	5-years OS rate (%)	5-years DFS rate (%)
LI (Ki67)≤50%	116	41.8	40.5
LI (Ki67)>50%	17	24.4	21.7
LI (MCM2)≤50%	57	48.7	48
LI (MCM2)>50%	75	30.4	28.4
LI (PCNA)≤50%	91	44	42.8
LI (PCNA)>50%	42	28.1	26.4

PCNA) showed a distinct division between lesions of dysplasia and carcinoma. The mean MCM2 LI values were 4% for normal mucosa and 15% for hyperplasia, compared with 40% for dysplasia and 60% for carcinoma, with the MCM2 LI being significantly greater than those of Ki67 and PCNA in dysplasia (P<0.05), and GCC (P<0.001). Strangely, significant differences were found between normal and hyperplasia but not between dysplasia and GCC for the LI of Ki67 and PCNA, which was contrary to the results of MCM2. Furthermore, the LIs of the three markers gradually increased with the progression of malignancy and poor degree of cell differentiation. The LI of Ki67 was always lower than those of MCM2 and PCNA in the different lesions and the LI of MCM2 was significantly higher than that of PCNA in poorly differentiated lesions such as dysplasia (p<0.05) and poorly differentiated GCC (p<0.05).

IHC expression of Ki67, MCM2, PCNA and clinically pathological features in follow-up of GCC patients

Demographic and pathological details of the patient cohort are included in Table 6. A total of 133 patients were recruited for this study; 47 cases were withdrawn due to failure in the follow up. The mean age of the patients was 60 years (range, 35~81 years), 110 patients were male and 23 female. The patients were classified with respect to their T (primary tumor) value; 7 cases were placed in T1, 53 cases in T2, 64 cases in T3 and there were 9 cases in T4. Histopathologically, 91 cases exhibited regional lymph node invasion and 2 cases had distant metastasis. According to TNM staging, 6 patients were classified as stage I, 28 patients as stage II, 63 patients as stage III and 36 patients as stage IV (Table 6).

A significant association is demonstrated between the LI of MCM2, LI of PCNA, and TNM stages, lymph node metastases and grade of gastric cardiac cancer (P<0.05). The LI of MCM2 and LI of PCNA was seen to increase with the TNM stage going from II to III to IV and these increases were statistically significant between stage II and stage III, as well as between stage II and stage IV (P<0.05). MCM2 expression in GCC was much higher than Ki67 and PCNA expressions. Furthermore, there was a clear association of increasing MCM2 LI

Table 8. Multivariate analysis result of prognostic factors by COX model.

Effect Factors	B	Wald	p value	OR	95% CI for OR	
					Lower	Upper
TNM Clinical Staging	0.707	22.226	<0.001	2.03	1.512	2.721
LI (MCM2)	1.23	6.354	0.012	3.42	1.315	8.9
Gender	0.642	6.064	0.014	1.9	1.14	3.169
Chemotherapy or not	0.695	5.588	0.018	0.5	0.281	0.888

B: coefficient; Wald: Wald test value.

with each increment of increasing grade (P<0.05) and this trend was also seen, although to a lesser degree, with Ki67 and PCNA (Table 6). When TNM staging progressed from II to III and to IV, the MCM2 LI also increased from 43% to 56% and to 61%, respectively (P=0.01). The same incremental trend was found with respect to regional lymph node metastasis (P=0.02, Table 6). There was no statistically significant correlation between MCM2 LI and tumor sizes (P=0.73). In addition, there was no significant correlation between IHC expression of MCM2 and gross morphology or pathology types (Table 6).

Correlation of Ki67, MCM2 and PCNA IHC expressions with prognosis in GCC patients

Details of IHC expression of three markers to survival are analyzed, where the 133 follow-up patients were divided into two groups according to their LI values. LI≤50% is the low expression group and LI>50% is the high expression group. Kaplan-Meier survival curves for Ki67, MCM2 and PCNA are shown in Fig. 4. For each individual protein, the result of the associated log-rank test suggests strong evidence that increased levels of expression are associated with reduced OS rate (P<0.05) and DFS rate (P<0.05). Fig. 4c, d show OS and DFS rates after curative resection in MCM2 LI-high and MCM2 LI-low patients. Significant differences in OS and DFS rates between MCM2 LI-high and MCM2 LI-low patients are shown in Table 7. The MCM2 LI-high patients had 1-, 3- and 5-year OS rates of 81%, 46% and 33% respectively, which was significantly worse than that of patients in MCM2 LI-low group (92%, 76%, and 55% respectively) (P<0.001). The corresponding DFS rates of patients in MCM2 LI-high group were 72%, 40% and 28%, respectively; significantly lower than those of patients in MCM2 LI-low group (90%, 72% and 54% respectively) (P<0.001). The difference in DFS rates between the two groups occurred mainly during the first 6 months after resection. However, OS rates in the two groups started to diverge 12 months after resection and became more prominent as the follow-up period increased. Also, Ki67 and PCNA staining show statistical difference of survival between the high and low groups (P<0.05, Fig. 4a,b,e,f, Table 7).

A multivariate (Cox regression) survival analysis was performed to determine if the markers studied here offered any prognostic information which would be significantly more valuable than that gained from the classical markers, such as gender, age, chemotherapy, TNM stage, T stage, N stage, LI (Ki67), LI (MCM2) and LI (PCNA). We found that the significant and independent prognostic factors in this analysis included TNM stage (P<0.001, OR=2.16), which had the greatest prognostic power, followed by LI (MCM2) (P=0.01, OR=3.54), gender (P=0.017, OR=1.849) and chemotherapy (P=0.018, OR=0.499). The influence of various prognostic factors on OS after curative resection is shown in Table8. Although Ki67 and PCNA were also significant prognostic factors, there was no significant improvement in prognostic value when these were included separately in the multivariate analysis.

Discussion

MCM2 expression, generally at basal and parabasal layers of normal cardiac glandular mucosa, had increased and extended to the multiple layers in hyperplasia and was distributed diffusely in the surface layer in dysplasia. The expression showed high sensitivity and specificity in marking of proliferative cells and in cells with proliferative potentials. Moreover, the level of MCM2 expression was inversely correlated with the differentiation grade of GCC, which is the same result as in gastric cancer (Tokuyasu et al., 2008; Czyzewska et al., 2009; Shomori et al., 2010; Giaginis et al., 2011). Compared with Ki67 and PCNA, the LI of MCM2 protein was higher, which suggests that it is a better marker with respect to the extent of cell proliferation, and also the different differentiation stages of gastric cardiac cancer. Furthermore, MCM2 was specifically expressed in all malignant cells and the same was trye for the most surface cells in dysplasia. These cells are readily accessible and can be obtained relatively easily and non-invasively, allowing precise early detection and diagnosis of GCC and precancerous lesion combined with traditional cytomorphological methods by HE staining.

Ki67 was first used as biomarker for dysplasia and malignancy in non-Hodgkin's lymphoma (Gerdes et al., 1984) and is now in wider use. Although it is as a good indicator in some cancer types (Keshgegian et al., 1998),

it is not so reliable for a number of others (Hilton et al., 1998). In fact, Ki67 antigen is not directly involved in cell cycle regulation but may be involved in ribosome biosynthesis during cell proliferation (MacCallum and Hal 2000). Thus, Ki67 may not be consistently expressed in cells that are entering G1 from Go (Gerdes et al., 1984), and in addition nutritional deprivation and dietary intake may also affect its expression (Baisch and Gerdes, 1987). PCNA is an auxiliary factor of DNA polymerase ‰, and is present at times of DNA repair (Tosch and Bravo, 1988). Therefore, PCNA staining does not always correlate with proliferation (Hall et al., 1990) and has also been detected in various quiescent cell populations. In contrast to Ki67 and PCNA, we know that the MCM proteins (MCM2-7) play an essential positive role in regulating eukaryotic DNA replication (Bell and Dutta, 2002). Being an integral heterohexamer component of the pre-replicative complex, they not only initiate DNA replication and control its amplification, limiting it to only once per cell cycle, but they also function as a helicase by unwinding DNA from its supercoiled state at replication forks (Stillman, 1996; Laskey and Madine, 2003). The MCM proteins were abundant in all phases of the cell cycle but were degraded in G0 cells, where cells are quiescent, senescent or differentiated (Musahl et al., 1998; Stoeber et al., 2001). Ever since Williams et al. (1998) showed that positive expressions of MCM2 and MCM5 in surface layer cells correlated with dysplasia severity, researchers have been trying to use MCM staining combined with surface sampling techniques to detect dysplasia and malignancy. Some investigations have achieved promising results for screening of cervical (Williams et al., 1998), bladder (Stoeber et al., 1999) and colon cancers (Davies et al., 2002), where expression of MCMs were found to be strictly confined to proliferative compartments of both normal and malignant tissues. In esophageal adenocarcinoma (AC), surface expression of MCM2 has been recommended to detect dysplasia and cancer (Sirieix et al., 2003). Also the LI of MCM2 was recently found to have significant associations with tumor, lymph node, and metastatic statuses, as well as pathological grade and histological grade for esophageal squamous cell carcinoma (ESCC) screening (Going et al., 2002; Kato et al., 2003).

Our study showed that MCM2 can be detected in almost all GCC samples and in only a minority of corresponding normal tissues, and it was consistently a better biomarker for cell proliferation than either Ki67 or PCNA, also exhibiting a significant correlation with tumor grade, which is consistent with some former reports (Todorov, 1998; Freeman et al., 1999; Rodins et al., 2002; Davidson et al., 2003). As it invades spontaneously and frequently recurs, the progression of GCC is unpredictable, making monitoring and diagnosing very difficult. Our results showed that GCC patients with high levels of MCM2 had poor post-surgical prognoses and that MCM2 was an independent predictor of disease-free survival. A number of other studies have shown MCM2 to be a valuable marker of patient survival in other forms of cancers (Ramnath et al., 2001; Tan et al., 2001; Wharton et al., 2001). Interestingly, in those studies, it was the maximal proliferative index (>50%) designated by the anti-MCM2 antibody as opposed to MCM2 that was indicative of survival. Ki67 and PCNA labeling, on the other hand, were not significantly different between the groups.

IHC is a widely accepted and well-documented method for characterizing patterns of protein expression. Using this method, we found that increased MCM2 IHC expression had a negative effect on both progression-free and overall survival in GCC patients after surgical resection. In multivariate analysis, age, grading, and tumor invasion depth were also found to be associated with prognosis concerning OS. In order to be adopted for routine clinical use, any new molecular markers need to enhance and be superior to the current routine estimators of prognosis. Thus, studies that do not extend statistical analyses beyond univariate survival measures are less valuable. In our univariate analysis, LI (Ki67) and LI (PCNA) were identified as poor prognostic factors for overall and tumor-free survival, and they were not significant in multivariate model with other, more highly associated co-variates. In contrast, MCM2 was found to be a better prognosis marker, correlating well and more significantly with the other important variables.

Because the measurement of MCM2 IHC expression may estimate the natural course of cardiac cancers, it may be used in screening of patients who have a poor prognosis. Furthermore, measurement of MCM2 may also provide molecular staging information concerning which can be used to decide if/when to initiate intensive surgical intervention or chemotherapy. Research work directed towards blocking the activation of MCM is underway in an attempt to find new ways of combating cancers. If new therapeutic methods which inhibit MCM expression are developed, it would be possible to treat cancers in more effective ways, for example by accelerating apoptosis and/or controlling the cell cycle events.

The present study indeed had several limitations. Firstly, all clinical data, such as recurrence and survival, were gathered by retrospective investigation; second, the size of the study population (n=133) puts a limitation on the power of the study. So a larger prospective study including analysis of IHC is warranted.

In summary, MCM2 is shown to be a more sensitive and specific biomarker than Ki67 and PCNA in indicating proliferation, detecting dysplasia and in diagnosing GCC. The present study validates the prognostic value of MCM2 and identifies increased MCM2 IHC expression as a negative factor in both disease-free and overall survival in patients with GCC. These findings could have important implications for the optimal routine clinical use.

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