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• GASTRIC CANCER •

Elevated level of spindle checkprotein MAD2 correlates with cellular mitotic arrest, but not with aneuploidy and clinicopathological characteristics in gastric cancer

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Abstract

AIM: To study the relevance of spindle assembly checkprotein MAD2 to cellular mitotic status, aneuploidy and other clinicopathological characteristics in gastric cancer.

METHODS: Western blot analyses were performed to analyze the protein levels of MAD2 and cyclin B1 in the tumorous and adjacent nontumorous tissues of 34 gastric cancer patients. Cell cycle distribution and DNA ploidy of cancer tissues were also determined by flow cytometry. Conventional statistical methods were adopted to determine the relevance of abnormal MAD2 level to mitotic status, aneuploidy and clinicopathological parameters.

RESULTS: Out of 34 gastric cancer patients 25 (74%) exhibited elevated MAD2 levels in their tumorous tissues compared with the corresponding nontumorous tissues. Elevation of MAD2 levels significantly correlated with the increased levels of cyclin B1 expression and G_2/M-phase distribution (P = 0.038 and P = 0.033, respectively), but was not relevant to aneuploidy. The gastric cancer patients with elevated MAD2 levels showed a tendency toward better disease-free and overall survival (P>0.05). However, no association was found between elevated MAD2 levels and patients' clinicopathological characteristics.

CONCLUSION: Elevation of MAD2 level is present in 74% of gastric cancer patients, and correlates with increased mitotic checkpoint activity. However, elevation of MAD2 level is not associated with patients' aneuploidy and any of the clinicopathological characteristics.

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INTRODUCTION

During the cell division cycle, the localization and segregation of chromosomes are under the surveillance of one group of proteins, called spindle assembly checkproteins[1-3]. Mitotic arrest-deficient proteins (MADs) and budding uninhibited by benzimidazole proteins (BUBs) are the major members of spindle assembly checkproteins[4-6]. Among them, MAD2 is a key component of MAD/BUB complex that can censor mis-segregation of chromosomes by monitoring the microtubule attachment and tension[4,7,8]. MAD2 is usually expressed at a high steady-state level and distributed at unattached kinetochores[9,10]. Re-localization of MAD2 along microtubules to the spindle poles is achieved by minus-end-directed dynein-dynactin complex only when all kinetochores properly attach to microtubules[10]. Once misaligned chromosomes or even a single unattached kinetochore is present, sufficient MAD2 molecules are kept in kinetochores to inhibit the onset of anaphase until all chromosomes exhibit proper bipolar attachment to the spindle. The kinetochore MAD2 can associate with and thus prevent the activation of anaphase-promoting complex (APC)[7,11-14]. APC is a kinetochore-localizing, CDC27-based ubiquitin ligase responsible for cyclin B1 degradation and in turn down-regulation of cyclin B1-associated CDC2 kinase activity, which is required for metaphase-anaphase transition and for exit from mitosis[7,11-14]. On the other hand, the microtubule-interfering agents, such as paclitaxel and nocodazole, can also elicit the spindle assembly checkpoint activity of MAD2[4,15,16]. In paclitaxel-treated cells, MAD2 mediates inhibition of APC's ability to ubiquitinate cyclin B1, which avoids the degradation of cyclin B1 and thus leads the cyclin B1/CDC2 activity to sustain longer[15]. This persistence of MAD2 and cyclin B1/CDC2 activation renders cells unable to exit from the metaphase and ultimately leads cells to apoptosis[15].

As described above, the role of MAD2 in spindle checkpoint machinery has been evidenced in many cell line studies. Clinically, it was reported that MAD2 was rarely the target for genetic alterations in digestive tract cancers[17,18]. Whatever from clinical investigation or animal models, the evidence demonstrating the relevance of MAD2 to cellular mitotic status or other histopathological characteristics is yet lacking. In this study, we investigated the level of MAD2 in 34 gastric cancer patients. The MAD2-related mitotic checkpoint activity was measured by cyclin B1 expression level and cell cycle G_2/M-phase fraction. Our data indicated that 25 out of 34 (74%) gastric cancer patients exhibited elevated MAD2 levels in their tumorous tissues rather than nontumorous tissues. Elevation of MAD2 level correlated with increased mitotic checkpoint activity but was not relevant to aneuploidy (chromosomal numerical alteration). Although the gastric cancer patients with elevated MAD2 levels exhibited a tendency toward better disease-free and overall survival, no correlation was found between abnormal MAD2 level and patients' clinicopathological characteristics.

MATERIALS AND METHODS

Patients and tumor specimens

Thirty-four primary gastric cancer tissues and their corresponding

normal mucosa were obtained from patients at Taipei Veterans General Hospital. The patients consisted of 25 men and 9 women (aged 43-80 years; mean: 63.8 years). Informed consent was obtained from each patient. All specimens were snap-frozen immediately after resection and stored at -80 ℃ until use. Parts of the specimens were taken for protein extraction and DNA content determination, and the remaining tissues were fixed in 40 g/L buffered formaldehyde for histologic examination. Hematoxylin and eosin staining of tissue sections was adopted to categorize the tumors according to the classification of Lauren[19].

Tissue lysate preparation and Western blot analysis

Tissue lysates were prepared by the method described previously[20]. Briefly, tumor and non-tumor specimens were ground down into powder in the presence of liquid nitrogen. Around 0.5 g of tissue powder was resuspended in 1.5 mL of 10 mmol/L Tris-Cl, pH 7.8, 140 mmol/L NaCl, 5 g/L deoxycholate, 10 mL/L NP-40, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin. The suspension was subjected to homogenization and further sonication on ice, and finally was ultracentrifuged at 100 000 *g* for 1 h at 4 ℃. The supernatant was saved and assayed for protein concentration (Bradford method). Aliquots (30 μg protein) of tissue lysates were separated on 100 g/L SDS-polyacrylamide gels, and electrotransferred onto polyvinylidene difluoride membranes. After blocked with PBST (phosphate-buffered saline plus 1 mL/L Tween-20) plus 50 g/L fat-free milk, the membranes were incubated with anti-MAD2, cyclin B1, and β-tubulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively, in PBST plus 50 g/L milk at 4 ℃ for 12 h. The membranes were then washed three times with PBST buffer, and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washed three times with PBST buffer, the protein bands were detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA).

Flow cytometric analysis of DNA content

The DNA ploidy and cell-cycle phase distribution of tissue specimens were measured by flow cytometric analysis[21]. Frozen specimens were first minced into 2 to 5 mm^3 pieces and further digested into single cell suspensions[22]. Cell suspensions were fixed with 800 mL/L ethanol at -20 ℃ at least for 30 min before subsequent Triton X-100 permeabilization and propidium iodide staining[16]. The cellular DNA content was analyzed using a FACStar flow cytometer with an argon laser tuned to the 488-nm line for excitation (BD Biosciences, San Jose, CA, USA).

Statistical analyses

Data were analyzed by χ^2 or *t* test. Survival rate was calculated by the Kaplan-Meier method. Statistical comparisons were made with Logrank test. The difference was considered to be significant when *P* value was less than 0.05.

RESULTS

Elevated MAD2 level occurs in human gastric cancer

Western blot analysis was performed to analyze the MAD2 expression level of the tumorous and adjacent nontumorous tissues of 34 gastric cancer patients. As shown in Figure 1, the MAD2 protein was detected in both tumorous and nontumorous tissue lysates. In most patients, MAD2 seemed labile in the nontumorous tissues rather than the tumorous tissues. The differential MAD2 level was confirmed by comparison with the levels of β-tubulin in the same-paired tissue lysates. A patient with elevated MAD2 level was defined as one whose MAD2 level in the tumorous tissue was higher than that in the adjacent nontumorous tissue, and elevated MAD2 level could be found in 25 of 34 (74%) cases of human gastric cancer.

Elevated MAD2 level correlates with increased mitotic arrest but not aneuploidy

The MAD2-related mitotic arrest was measured by cyclin B1 level and cell cycle G_2/M-phase fraction. We found that 18 of 34 (53%) gastric cancer patients had elevated cyclin B1 expression level in their tumorous rather than nontumorous tissues (three examples shown in Figure 1). There was a statistically significant correlation between elevated MAD2 level and elevated cyclin B1 level ($P = 0.038$), as 16 of 25 (64%) gastric cancer patients who had elevated MAD2 levels also manifested higher levels of cyclin B1 in their tumorous tissues (Table 1). Moreover, the DNA contents of tumor specimens of 29 patients were successfully determined by flow cytometric analysis. The data presented by mean±SD (%) of phase fractions are shown in Table 2. We observed that the ratio of G_2/M-phase fraction in the tumor specimens exhibiting elevated MAD2 levels was statistically higher than that in the tumorous tissues with a normal MAD2 level (10.6 ± 4.9% *vs* 6.4 ± 4.0%, $P = 0.033$). No significant difference in the ratios of G_0/G_1 and S-phase fractions was found between the tumors with or without elevated MAD2 levels (Table 2). In addition, DNA ploidy was also determined from the tumor specimens of 32 patients. Although 18 of 32 (56%) patients were found to have aneuploid tumor cells, no correlation was observed between the occurrence of aneuploidy and elevated MAD2 level in cancer tissues ($P = 1.000$, Table 3).

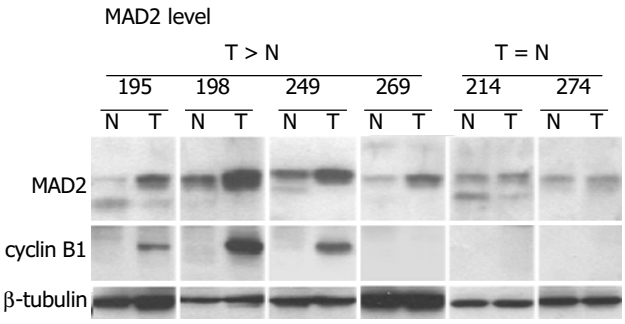

Figure 1 Examples of elevation of MAD2 and cyclin B1 levels in human gastric cancer. Western blot analyses were performed to analyze the protein levels of MAD2 and cyclin B1 in the lysates from non-tumorous tissues (N) and tumorous tissues (T) of gastric cancer patients, #195, #198, #249, #269, #214, and #274. The levels of β-tubulin in the same-paired tissue lysates were analyzed as internal control.

Table 1 Cyclin B1 expression status of 34 gastric cancer tissues with or without MAD2 overexpression

	Cyclin B1 level	
	T > N	T = N
MAD2 level		
T > N ($n = 25$)	16	9
T = N ($n = 9$)	2	7
	$P = 0.038$	

T: tumorous tissue; N: non-tumorous tissue.

Table 2 Cell cycle phase fractions of 29 gastric cancer tissues with or without MAD2 overexpression (mean±SD)

	Phase fraction (%)		
	G_0/G_1	S	G_2/M
MAD2 level			
T > N (n = 20)	80.8±5.9	8.6±7.0	10.6±4.9
T = N (n = 9)	83.5±7.4	9.9±7.7	6.4±4.0
	P = 0.301	P = 0.657	P = 0.033

T: tumorous tissue; N: non-tumorous tissue.

Table 3 DNA ploidy status of 32 gastric cancer tissues with or without MAD2 overexpression

	Diploidy	Aneuploidy
MAD2 level		
T > N (n = 23)	10	13
T = N (n = 9)	4	5
	P = 1.000	

T: tumorous tissue; N: non-tumorous tissue.

Elevated MAD2 level does not correlate with clinicopathological characteristics

The relationship of elevated MAD2 level with clinicopathological characteristics was also investigated and summarized (Table 4). The evaluated parameters included age at diagnosis, tumor site and size, cell differentiation grade, stromal reaction, invasive and metastatic status, *etc*. For the 34 studied patients, age and gender did not associate with higher levels of MAD2 in cancer tissues (P>0.05). There was no association between elevated MAD2 levels and different tumor sites (upper, middle, lower or whole stomach), tumor sizes, and other histopathological characteristics including grade of cell differentiation, Borrmann type, stromal reaction (medullary, intermediate or schirrhous type), infiltration type (α, β or γ), Lauren histological classification (intestinal or diffuse type), and TNM staging (I-IV), either. In addition, elevation of MAD2 level in cancer tissues was not correlated with the invasion parameters, including the lymphatic duct or vessel invasion and depth of cancer invasion (mucosa, submucosa, propria muscle, subserosa, serosa, serosa exposed), and metastatic status such as peritoneal dissemination and lymph node or liver metastasis (Table 4). Finally, the patients with elevated MAD2 levels in tumor tissues exhibited higher five-year overall and disease-free survival rates in comparison with those without elevated MAD2 levels (48.0% *vs* 20.8% and 46.3% *vs* 11.1%, respectively), but the difference did not reach a significant level (P = 0.478 and 0.229, respectively; Table 4).

Table 4 Relationships between elevated MAD2 levels and clinicopathological characteristics (mean±SD)

	MAD2 level		
	T>N (n = 25)	T=N (n = 9)	P
Age (yr)	62.8±10.3	66.4±5.0	0.325
Sex (male/female)	19/6	6/3	0.586
Site of tumor			0.828
Upper stomach	4	2	
Middle stomach	6	2	
Lower stomach	14	4	
Whole stomach	1	1	
Size of tumor (cm)	7.3±2.3	8.0±2.9	0.470
Grade of cell differentiation			0.146
Well differentiated	1	0	
Moderately differentiated	13	8	
Poorly differentiated	11	1	
Borrmann type			0.664
0	2	0	
1 + 2	6	2	
3 + 4	17	7	
Stromal reaction			0.739
Medullary type	6	3	
Intermediate type	12	3	
Schirrhous type	7	3	
Infiltration type			0.475
α	4	2	
β	8	1	
γ	13	6	
Lauren histological classification			0.448
Intestinal type	13	3	
Diffuse type	12	6	
Lymph node metastasis (Yes/No)	15/10	6/3	1.000
Lymphatic duct invasion (Yes/No)	18/7	7/2	1.000
Vascular invasion (Yes/No)	2/23	1/8	1.000
Liver metastasis (Yes/No)	1/24	0/9	1.000
Peritoneal dissemination (Yes/No)	2/23	1/8	1.000
Depth of cancer invasion			0.738
Mucosa, submucosa	1	0	
Propria muscle, subserosa	5	1	
Serosa	18	8	
Serosa (infiltration) exposed	1	0	
TNM stage			0.932
I	3	1	
II	8	2	
III	8	3	
IV	6	3	
Five-yr overall survival rate	48.0%	20.8%	0.478
Five-yr disease-free survival rate	46.3%	11.1%	0.229

DISCUSSION

Spindle assembly checkpoint is one of the mechanisms to guard the fidelity of cell division cycle[1-3]. MAD2 is a key component of spindle assembly checkprotein complex MAD/BUB that is responsible for monitoring the localization and segregation of chromosomes[4,7,8]. MAD2 could induce mitotic arrest by associating with and thus inhibiting APC when microtubule-interfering agents were present in cancer cell cultures[4,15]. However, the evidence demonstrating the clinical relevance of MAD2 to cancer cell mitotic status is yet lacking. In this study, we provided the clinical data to support the mitotic checkpoint role of MAD2 in cancer tissues. We found that 74% of our gastric cancer patients had elevated levels of MAD2 in their tumorous tissues. These patients also exhibited more cyclin B1 expression and G_2/M-phase distribution in their cancer cells. Because MAD2 can interfere with APC and APC is an ubiquitin ligase responsible for cyclin B1 degradation, elevation of both cyclin B1 expression and cellular G_2/M-phase ratio may be resulted from a higher mitotic checkpoint activity that is expected of elevated MAD2 level. Noteworthily, these patients had a tendency toward longer disease-free and overall survival. We speculate that the checkpoint activity of MAD2 exerted in these patients monitors the interaction of chromosomes with spindle fibers, which is finally linked with better disease-free and overall survival. Elevated level of MAD2 seems to be a possible target

for potential development of novel therapeutic or prognostic modalities in the future.

Our data indicate that elevated MAD2 levels did not prevent the occurrence of aneuploidy in gastric cancer. Aneuploidy is one of the hallmarks of cancer cells[23-25]. Considering spindle assembly checkproteins function as a monitor for the fidelity of chromosomal segregation, impairment of spindle assembly checkpoint is expected to associate with the development of cancer cell aneuploidy. However thus far, many aneuploid cancer cell lines did undergo mitotic arrest in response to spindle damage, indicating that not all cancer cells with aneuploidy had an impaired spindle checkpoint[26-28]. Moreover, accumulating studies have demonstrated that the BUBs (BUB1, BUBR1 and BUB3) and MADs (MAD1 and MAD2) were rarely the targets for genetic alterations in a variety of human cancer types including head-and-neck squamous cell carcinoma[29], non-small cell lung cancer[29,30], thyroid follicular neoplasms[28], hepatocellular carcinoma[27], and digestive tract cancers[17,18,30]. These data suggest that cancer cell aneuploidy may arise from the alternative defects yet to be discovered. Despite of the low frequency of gene mutation, a research of 43 gastric cancer patients concluded that overexpression of BUB1, BUBR1 or/and BUB3 was observed in >60% of cases[31]. There was no statistically positive correlation between overexpression of BUBs and cancer aneuploidy. Instead, the overexpression was significantly correlated with Ki-67 expression of tumor cells, suggesting that BUBs are proliferation-associated proteins other than spindle checkproteins in gastric cancer[31].

The gastric cancer patients with different molecular alterations were shown to have distinct histopathological features. For example, simultaneous overexpression of hepatocyte growth factor receptor (c-Met), autocrine motility factor receptor (AMFR) and urokinase-type plasminogen activator receptor (uPAR) was correlated with positive lymphatic vessel invasion and infiltration[32]. Estrogen receptor (ER) was more expressed in diffuse-type patients with regional lymph node metastasis[33]. Additionally, positive expression of nm23 was detected in as high as 74% of gastric cancer patients and was related to patients' age, tumor size, Borrmann type, Lauren classification, and TNM stage[34]. COX-2 overexpression significantly correlated with TNM staging; while abnormal expression of E-cadherin/β-catenin complex occurred more significantly in Borrmann types III/IV than in types I/II. In our present study, no histopathological parameter was found to be associated with elevated MAD2 level in gastric cancer patients. It was reported consistently that MAD2 was significantly overexpressed in colorectal adenocarcinoma, but was not related to differentiation or other clinical parameters.

In conclusion, an elevation of spindle checkprotein MAD2 level was observed in 74% of our gastric cancer patients, and was significantly correlated with the increased levels of cyclin B1 expression and G_2/M-phase distribution in cancer tissues. However, an elevated MAD2 level was not associated with aneuploidy and other clinical factors, including demographic features and histopathological characteristics.

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