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BubR1 and *AURKB* overexpression are associated with a favorable prognosis in gastric cancer

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Abstract. The majority of human solid tumors exhibit aneuploidy caused by impairment of the mitotic checkpoint. Since the Mad2, BubR1 and Aurora kinase B (AURKB) proteins are involved in the mitotic checkpoint, we investigated *Mad2*, *BubR1* and *AURKB* mRNA expression and its effect on clinicopathological parameters and prognosis in 100 consecutive patients who underwent surgical resection for gastric cancer. *Mad2*, *BubR1* and *AURKB* mRNA expression levels in gastric cancer tissues and corresponding normal gastric mucosa were compared by real-time quantitative RT-PCR. The data were then correlated to clinicopathological parameters and prognosis. The expression of *Mad2*, *BubR1* and *AURKB* mRNA was found to be significantly higher in cancer tissue compared to normal tissue. *BubR1* and *AURKB* expression was significantly higher during the earlier stages of the disease. Patients with high *BubR1* expression had improved relapse-free survival and overall survival compared to patients with low *BubR1* expression. Multivariate analysis of stage II and III patients indicated that high expression of *BubR1* and/or *AURKB* was associated with improved overall survival. We conclude that overexpression of *BubR1* and *AURKB* is associated with a low risk of gastric cancer progression, and that overexpression of *BubR1* and/or *AURKB* can therefore be used to identify gastric cancer patients with a favorable prognosis.

Introduction

Despite the declining incidence of gastric cancer, the disease remains the fourth most common cancer and the second most frequent cause of cancer-related death worldwide (1,2). Recent progress in diagnostic and treatment technologies has improved the long-term survival of patients with early-stage gastric cancer, although the prognosis for patients with advanced disease remains unfavorable (3). Surgical treatment is the mainstay of therapy for patients with localized disease, but adjuvant chemotherapy is required after surgical resection in advanced cases (4). Thus, the identification of prognostic factors may contribute to improved treatment strategies for gastric cancer patients. This requires further insight into carcinogenesis and cancer progression.

The majority of human solid tumors exhibit aneuploidy (5), which is a very early event in the progression of gastric cancer (6). Tumor cells become aneuploid as a result of aberrant mitotic division, caused by a defective mitotic checkpoint response. The mitotic checkpoint is a signaling cascade that arrests the cell cycle in mitosis when even a single chromosome is not properly attached to the mitotic spindle (5,7).

The mitotic checkpoint complex contains the anaphase-promoting complex/cyclosome activator Cdc20, as well as mitotic checkpoint kinases (MCKs) such as Mad2 (mitotic arrest deficient-like 1; MAD2L1), BubR1 (budding uninhibited by benzimidazoles 1 homolog ß; BUB1B) and Bub3. The MCKs are regarded as effectors of the mitotic checkpoint. Within the MCK complex, both Mad2 and BubR1 directly bind Cdc20 (8). A large number of aneuploid cell lines do not appear to harbor mutations in the known mitotic checkpoint genes. It is thus possible that mitotic checkpoint dysfunction in these cell lines results from altered expression levels of the known checkpoint genes (9).

At the mitotic checkpoint, the chromosomal passenger complex lies at the top of a cascade that recruits other MCKs (10). The core chromosomal passenger complex is composed of AURKB (Aurora kinase B) and three non-enzymatic subunits, INCENP, survivin and borealin. The non-enzymatic members of the complex control the targeting enzymatic activity and stability of AURKB (11,12).

Aberrant expression of the MCKs or chromosomal passenger protein in mammalian cells leads to aneuploidy. Overexpression of Mad2, BubR1 or AURKB has been observed in human cancer cells (13-15), suggesting that this aberrant expression plays an important role in cancer initiation and progression. We hypothesized that mitotic checkpoint dysfunction is associated with gastric cancer. In the present study, *Mad2*, *BubR1* and *AURKB* mRNA expression was investigated in gastric cancer using real-time quantitative reverse transcription-

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Key words: mitotic checkpoint, chromosomal passenger, gastric cancer, clinicopathological parameter, survival

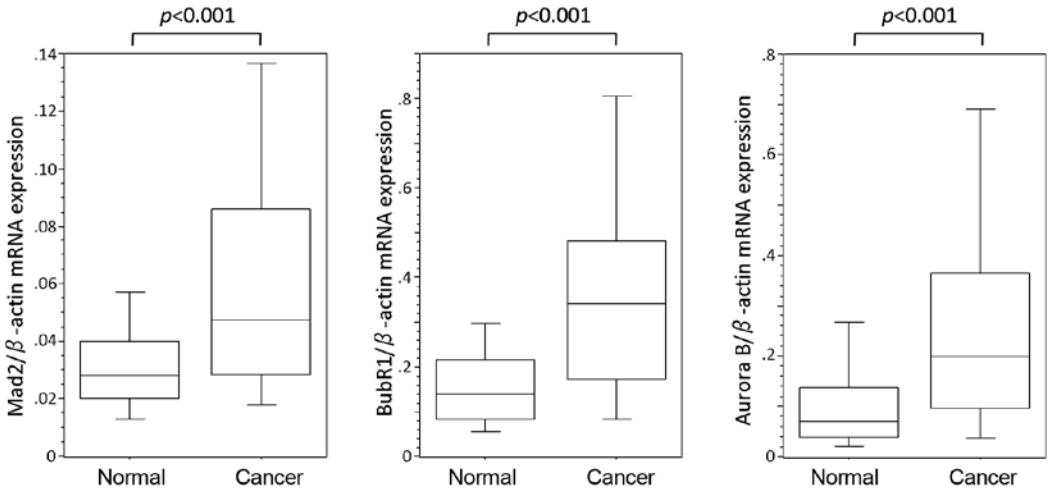

Figure 1. Mad2, BubR1 and AURKB mRNA expression levels in gastric cancer and normal gastric tissue.

polymerase chain reaction (RT-PCR). The findings were then correlated with clinicopathological parameters and prognosis.

Materials and methods

Patients and tissue samples. We studied 100 consecutive patients (81 males and 19 females; age range 34-92 years; median 69 years) who underwent surgical resection for gastric cancer at our institution between May 2004 and September 2007. Pathological classifications were made according to the TNM staging system (6th edition, 2002) of the International Union Against Cancer (UICC). Lymphatic and vascular invasion was regarded as negative when the findings were absent or slight, and positive when the findings were moderate or marked (Table I). Patients did not undergo pre-operative chemotherapy or radiotherapy, nor adjuvant chemotherapy following surgical resection. Stage IV patients received s-1-based systemic chemotherapy without any radiotherapy. All patients underwent a follow-up examination, with a median follow-up time at analysis of 19 months. During this period, there were 23 cases of recurrence, and 27 patients succumbed to the disease.

Immediately after surgery, a small piece of gastric cancer tissue and matched adjacent normal mucosa (taken from the borders of the surgical specimen) were separately placed directly in RNA stabilization reagent (RNAlater, Qiagen, Valencia, CA) and stored at -80˚C until further analysis.

The study was approved by the Institutional Review Board of the Tokyo Medical and Dental University. Written informed consent was obtained from all patients.

RNA extraction and cDNA synthesis. Total RNA for each sample was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The concentration of total RNA was determined by absorption measurements at 260 and 280 nm using a UV spectrophotometer (Beckman Coulter, Fullerton, CA). For cDNA synthesis, 10 μg of total RNA was reverse-transcribed into cDNA samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.

Real-time quantitative RT-PCR. Expression levels of *Mad2*, *BubR1* and *AURKB*, as well as of *β-actin* as the endogenous control, were determined by real-time quantitative PCR using the 7300 Real-Time PCR System (Applied Biosystems). TaqMan gene expression assays were purchased from Applied Bioystems (*Mad2*, Hs01554515_g1; *BubR1*, Hs01084828_m1; *AURKB*, Hs00177782_m1; *β-actin*, Hs99999903_m1). The PCR reaction was carried out using TaqMan Universal PCR Master Mix (Applied Biosystems) with 1 μl of cDNA in a 24-μl final reaction volume. Thermal cycling conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, 40 cycles of 15-sec denaturation at 95˚C, and 1 min of annealing at 60˚C. cDNA synthesized by HCT15 was used as the calibrator. Each sample was run in duplicate for both the target and endogenous genes. The amount of *Mad2*, *BubR1* and *AURKB* (target) normalized to the endogenous control and relative to the calibrator was determined by the comparative Ct method for relative quantification (ΔΔCt method) (16) using Relative Quantification Study Software (7300 Sequence Detection System version 1.4, Applied Biosystems).

Statistical analysis. The receiver-operating characteristic (ROC) curve was used to determine the cutoff value of each mRNA, as previously reported (17,18). Briefly, the ROC curve was constructed by plotting all possible sensitivity/1-specificity pairs resulting from continuously elevating the cutoff values. The optimal cutoff point gave the best combination of specificity and sensitivity according to the ROC curve. This cutoff value was determined using the Youden index (18), which is commonly used to measure overall diagnostic effectiveness. The ROC curve can be used to distinguish gastric cancer from normal controls.

Differences between groups were evaluated using the Wilcoxon signed-rank test and the χ^2 test. Correlation analysis was performed using Spearman's rank correlation. Overall survival curves and relapse-free survival curves were plotted according to the Kaplan-Meier method and measured from the day of surgery, with the log-rank test applied for comparisons. Prognostic factors were examined by univariate and multivariate analyses based on Cox's proportional hazards

Table I. Comparison of *Mad2*, *BubR1* and *AURKB* mRNA expression and clinicopathological parameters.

		Mad2			*BubR1*			*AURKB*		
Clinicopathological parameters	Total	Low	High	p-value	Low	High	p-value	Low	High	p-value
All cases	100	35	65		34	66		44	56	
Age										
>69	51	15	36		17	34		21	30	
≤69	49	20	29	0.232	17	32	0.886	23	26	0.562
Gender										
Male	81	31	50		26	55		16	65	
Female	19	4	15	0.157	8	11	0.407	5	14	0.527
Depth of invasion										
T1/T2	54	16	38		12	42		20	34	
T3/T4	46	19	27	0.224	22	24	0.007	24	22	0.129
Lymph node metastasis										
N0	30	6	24		7	23		8	22	
N1/N2/N3	70	29	41	0.040	27	43	0.141	36	33	0.022
Distant metastasis										
M0	88	32	56		26	62		38	50	
M1	12	3	9	0.439	8	4	0.011	6	6	0.656
Stage										
I	29	6	23		5	24		8	21	
II	22	9	13		8	14		11	11	
III	30	14	16		7	23		12	18	
IV	19	6	13	0.184	14	5	0.001	13	6	0.041
Lymphatic invasion										
Negative	48	17	31		10	38		19	29	
Positive	52	18	34	0.933	24	28	0.007	25	27	0.392
Vascular invasion										
Negative	31	11	20		10	21		15	16	
Positive	69	24	45	0.946	24	45	0.805	29	40	0.554
Histological type										
Differentiated	50	15	35		10	40		17	33	
Undifferentiated	50	20	30	0.295	24	26	0.003	27	23	0.044

model. Differences were considered significant at $p<0.05$. All analyses were performed with the statistical software package Stat View (version 5.0) (Abacus Concepts, Berkeley, CA).

Results

Expression of Mad2, BubR1 and AURKB in gastric cancer and determination of cutoff values. *Mad2*, *BubR1* and *AURKB* mRNA expression levels in cancer tissue and normal tissue were assayed by real-time RT-PCR, and were found to be significantly higher in cancer tissue compared to normal tissue ($p<0.001$, $p<0.001$ and $p<0.001$, respectively; Fig. 1). There was significant correlation between *Mad2* and *BubR1*, *Mad2* and *AURKB*, and *BubR1* and *AURKB* ($\varrho=0.424$, $p<0.001$; $\varrho=0.375$, $p<0.001$; $\varrho=0.619$, $p<0.001$; respectively; Fig. 2).

The cutoff values for *Mad2*, *BubR1* and *AURKB* were calculated using the ROC curve as 0.038, 0.244 and 0.170, respectively. Patients with cancer tissue values below the cutoff were considered to be in the low expression group, whereas those with cancer tissue values above the cutoff were placed in the high expression group. High Mad2 expression was noted in 65% (65/100), high BubR1 expression in 66% (66/100), and high AURKB expression in 56% (56/100) of patient tissue samples (Table I).

Correlation between clinicopathological factors and Mad2, BubR1 and AURKB mRNA expression. Table I shows the clinicopathological data and *Mad2*, *BubR1* and *AURKB* mRNA expression levels in the cancer tissue. *Mad2* was significantly associated with lymph node metastasis ($p=0.040$). *BubR1* was significantly associated with depth of invasion, distant metastasis, stage classification, lymphatic invasion, and histological type ($p=0.007$, 0.011, 0.001, 0.007 and 0.003, respectively). *AURKB* was significantly associated with lymph

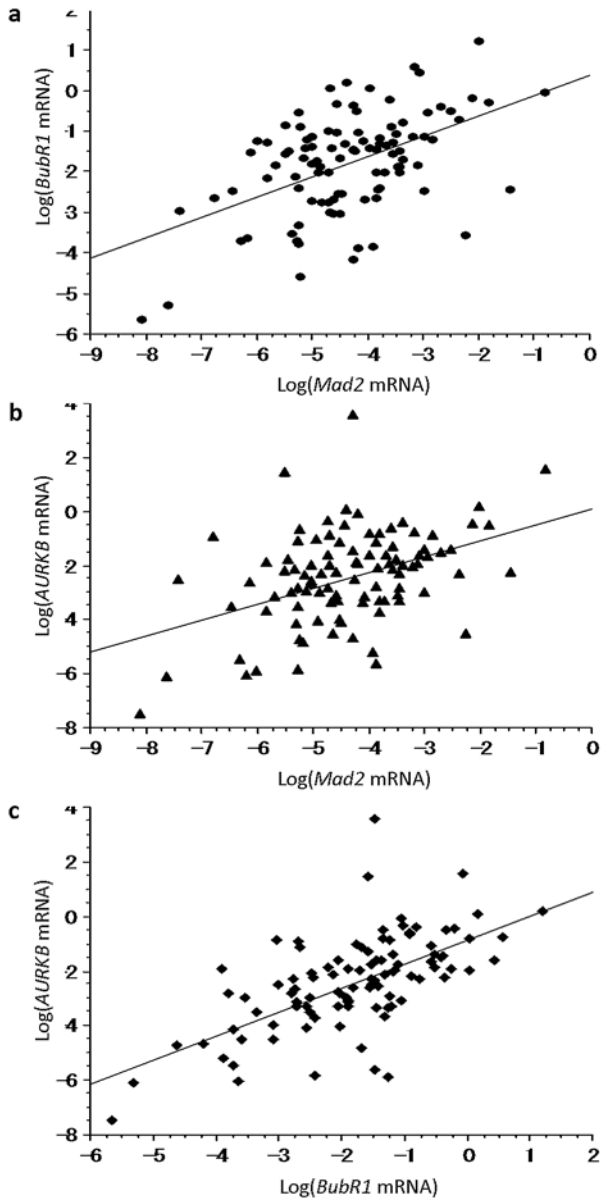

Figure 2. Correlation between mRNA levels of (a) Mad2 and BubR1 (ϱ=0.424, p<0.001), (b) Mad2 and AURKB (ϱ=0.375, p<0.001), and (c) BubR1 and AURKB (ϱ=0.619, p<0.001) in cancer tissue.

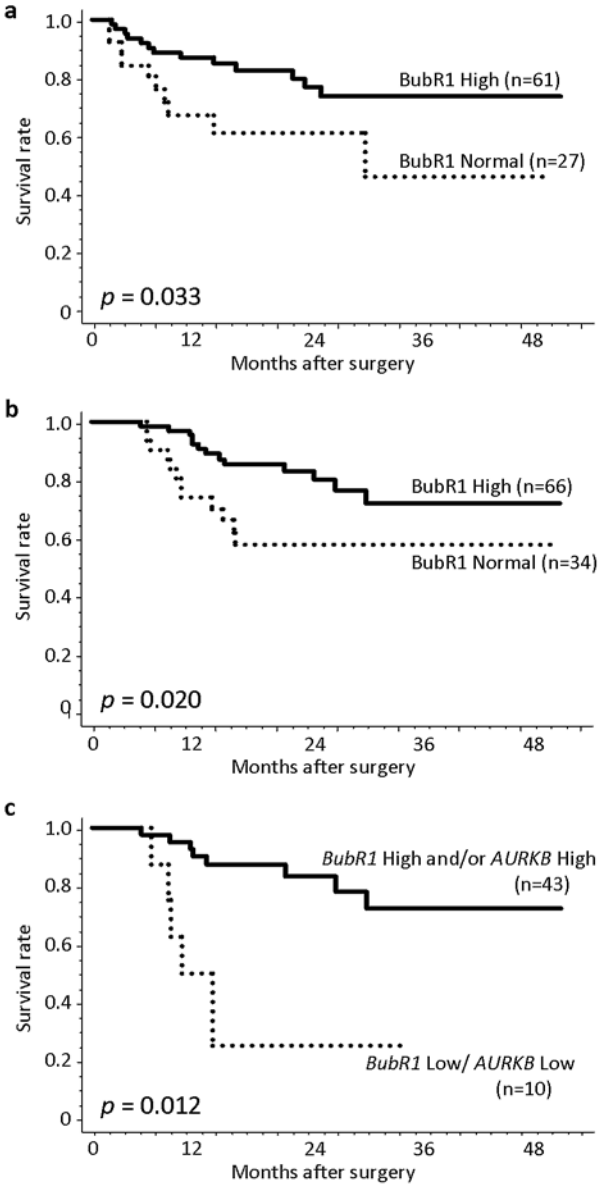

Figure 3. (a) Relapse-free survival of 88 patients who underwent a curative R0 resection in relation to the expression of BubR1 (p=0.033). (b) Overall survival of all 100 patients in relation to the expression of BubR1 (p=0.020). (c) Overall survival of 53 stage II and III patients in relation to the combined expression of BubR1 and AURKB (p=0.012).

node metastasis, stage classification and histological type (p=0.022, 0.041 and 0.044, respectively).

Prognostic value of Mad2, BubR1 and AURKB mRNA expression levels. Relapse-free survival (RFS) and overall survival (OS) were analyzed using the Kaplan-Meier method in relation to *Mad2*, *BubR1* and *AURKB* mRNA expression. The median survival of the 100 patients was 19 months. During this period, there were 23 cases (23%) of recurrence, and 27 patients (27%) succumbed to the disease. Patients with high *BubR1* mRNA expression had a significantly increased RFS and OS compared to those with low expression (p=0.033, Fig. 3a; p=0.020, Fig. 3b, respectively). No other factors were significantly associated with RFS and OS.

Among the stage II and III patients (n=53), there were 14 cases (26%) of recurrence, and 13 patients (25%) succumbed to the disease. *BubR1* mRNA expression was not associated with patient survival.

Survival was also analyzed in relation to the combination of *BubR1* and *AURKB* mRNA expression, since AURKB is a regulator of BubR1 during chromosome alignment at the metaphase (19). Four different subgroups were identified: low *BubR1*/low *AURKB* (n=10), high *BubR1*/low *AURKB* (n=14), low *BubR1*/high *AURKB* (n=6), and high BubR1/high *AURKB* (n=23). Patients with high expression of *BubR1* and/or *AURKB* had a more favorable outcome, though without significant differences between the three groups (data not shown). Consequently, these three groups were considered a single group. High *BubR1* and/or high *AURKB* expression was associated with significantly increased OS (p=0.012; Fig. 3c), but not RFS.

Table II. Univariate and multivariate analysis for relapse-free survival in patients who underwent R0 surgery (n=88).

	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	p-value	Hazard ratio	95% CI	p-value
Age						
>69	1	Reference				
≤69	1.72	0.73-4.06	0.216			
Gender						
Female	1	Reference				
Male	2.67	0.62-11.38	0.186			
Depth of invasion						
T1/T2	1	Reference		1	Reference	
T3/T4	6.96	2.58-18.80	0.001	5.15	1.21-21.96	0.027
Lymph node metastasis						
N0	1	Reference				
N1/N2/N3	2.13	0.79-5.74	0.135			
Stage						
I/II	1	Reference		1	Reference	
III/IV	4.35	1.71-11.07	<0.001	1.25	0.33-4.71	0.743
Histological type						
Differentiated	1	Reference		1	Reference	
Undifferentiated	2.37	1.00-5.59	0.049	1.1	0.42-2.87	0.846
Mad2 mRNA expression						
High	1	Reference				
Low	1.56	0.68-3.56	0.294			
BubR1 mRNA expression						
High	1	Reference		1	Reference	
Low	2.4	1.05-5.51	0.038	1.66	0.67-4.08	0.273
AURKB mRNA expression						
High	1	Reference				
Low	1.63	0.72-3.70	0.242			

Univariate and multivariate analysis of survival. Univariate and multivariate analyses of factors related to OS were performed for all patients (n=100). The same analyses of factors related to RFS were performed for patients who underwent R0 surgery (n=88). Univariate analysis for RFS revealed that depth of invasion, stage classification, histological type and *BubR1* mRNA expression level were significantly associated with patient survival (p=0.001, <0.001, 0.049 and 0.038, respectively; Table II). According to the multivariate analysis, depth of invasion was the only independent prognostic factor for RFS (p=0.027; Table II). In terms of OS using univariate analysis, depth of invasion, lymph node metastasis, stage classification and *BubR1* mRNA expression level were significantly associated with patient survival (p=0.002, 0.015, 0.009 and 0.025, respectively; Table III). In the multivariate analysis, lymph node metastasis was the only independent prognostic factor for OS (p=0.037; Table III). The expression level of *BubR1* mRNA was a significant predictor according to univariate, but not multivariate, analysis.

In stage II and III patients (n=22, n=31, respectively), according to univariate and multivariate analyses, the combination of *BubR1* and *AURKB* mRNA expression levels was an independent and significant prognostic factor for OS (p=0.024; multivariate analysis, Table IV), but not for RFS.

Discussion

We revealed a significant correlation between *Mad2*, *BubR1* and *AURKB* mRNA expression levels and clinicopathological factors in gastric cancer. In addition, the combination of *BubR1* and *AURKB* mRNA expression was identified as an independent prognostic factor. These findings suggest that *Mad2*, *BubR1* and *AURKB* play a crucial role in gastric cancer progression.

Aneuploidy is commonly observed in the majority of human solid tumors (20,21), including gastric cancer (6). While complete loss of the mitotic checkpoint is lethal in vertebrates, a weakened mitotic checkpoint is frequently noted in cancer

Table III. Univariate and multivariate analysis for overall survival in all patients (n=100).

	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	p-value	Hazard ratio	95% CI	p-value
Age						
>69	1	Reference				
≤69	1.31	0.59 - 2.93	0.504			
Gender						
Female	1	Reference				
Male	0.99	0.37-2.65	0.985			
Depth of invasion						
T1/T2	1	Reference		1	Reference	
T3/T4	4.19	1.67-10.50	0.002	3.69	0.74 - 18.49	0.112
Lymph node metastasis						
N0	1	Reference		1	Reference	
N1/N2/N3	11.89	1.61-87.99	0.015	9.69	1.14-82.29	0.037
Stage						
I/II	1	Reference		1	Reference	
III/IV	3.43	1.36-8.61	0.009	0.47	0.09-2.52	0.379
Histological type						
Differentiated	1	Reference				
Undifferentiated	2.29	0.99-5.30	0.054			
Mad2 mRNA expression						
High	1	Reference				
Low	1.51	0.68-3.32	0.310			
BubR1 mRNA expression						
High	1	Reference		1	Reference	
Low	2.46	1.12-5.41	0.025	1.82	0.81-4.07	0.147
AURKB mRNA expression						
High	1	Reference				
Low	1.31	0.60-2.87	0.505			

cells. It has been speculated that a weakened mitotic checkpoint contributes to aneuploidy and tumorigenesis without loss of viability (22). Aneuploid human cancers frequently exhibit altered expression of the mitotic kinases (including Mad2, BubR1 and AURKB) (21). Often, this takes the form of overexpression (20,23). The mitotic kinases must be tightly regulated, as both their reduced amounts and overproduction induce aneuploidy (5,14). Although *Mad2*, *BubR1* and *AURKB* have not been established as oncogenes by the standard criteria, overexpression of Mad2 in transgenic mice leads to a wide variety of neoplasias, and exogenous overexpression of AURKB in Chinese hamster embryo cells leads to chromosomal instability (24,25). In the present study, we detected overexpression of *Mad2*, *BubR1* and *AURKB* mRNA in gastric cancer.

Mad2, *BubR1* and *AURKB* mRNA expression levels were positively correlated with each other, suggesting that Mad2, BubR1 and AURKB are controlled by a common linking factor. Overexpression of *Mad2*, *BubR1* and *AURKB* was more frequently observed during the earlier stages of cancer development compared to advanced stages. These results suggest that overexpression of *Mad2*, *BubR1* or *AURKB* contributes to the initiation of tumorigenesis and, subsequently, the inhibition of gastric cancer progression. In a recent study, centromere protein E haplo-insufficient mice (which had a weakened mitotic checkpoint) exhibited increased frequency of spontaneous lymphomas and lung tumors. Unexpectedly, treatment with chemical tumor inducers inhibited tumorigenesis in these mice (26). These findings indicate that moderate levels of genetic instability promote cell growth and tumorigenesis, whereas high levels result in cell death and tumor suppression. The most surprising finding was the identification of a previously unsuspected role for aneuploidy in suppressing tumor growth (27). It is now widely accepted that gastric cancer develops through the accumulation of genetic alterations (28) that consist of abnormal chromosome numbers (e.g., aneuploidy, polyploidy) and structural changes (e.g., translocations, mutations) (29). Aneuploidy is a very early event in the progression of gastric cancer. The molecular mechanism that initiates and drives aneuploidy has not been identified. Several possibilities exist, such as defective sister chromatid cohesion or an abnormal

Table IV. Univariate and multivariate analysis for overall survival in stage II and III patients (n=53).

	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	p-value	Hazard ratio	95% CI	p-value
Age						
>69	1	Reference				
≤69	1.29	0.42-3.94	0.658			
Gender						
Female	1	Reference				
Male	0.95	0.21-4.31	0.942			
Depth of invasion						
T1/T2	1	Reference		1	Reference	
T3/T4	1.69	0.55-5.16	0.361	0.84	0.22-3.22	0.803
Stage						
I/II	1	Reference		1	Reference	
III/IV	1.45	0.44-4.76	0.538	1.87	0.47-7.40	0.370
Histological type						
Differentiated	1	Reference		1	Reference	
Undifferentiated	1.64	0.54-5.01	0.388	1.30	0.40-4.18	0.666
BubR1/*AURKB* mRNA expression						
Others	1	Reference		1	Reference	
Both low	4.30	124-14.90	0.021	4.64	1.23-17.56	0.024

kinetochore structure, but mitotic checkpoint dysfunction and centrosome abnormalities appear to play a more significant role in tumorigenesis (6). It is therefore reasonable to assume that a number of genetic alterations accompanied by overexpression of *Mad2*, *BubR1* and *AURKB* lead to high levels of aneuploidy, thereby preventing cancer progression in early-stage gastric cancer. Conversely, a number of genetic alterations without overexpression of *Mad2*, *BubR1* and *AURKB* lead to moderate levels of aneuploidy, thereby promoting progression in advanced-stage gastric cancer. Our results are in accordance with the view that aneuploidy and massive genetic instability play a role in cancer suppression.

Patients with high expression of *BubR1* had significantly increased RFS and OS. Unexpectedly, there was no correlation between *Mad2* and survival, although Mad2 and BubR1 have a synergistic effect on checkpoint function (30). It is highly probable that gastric cancer progression is more affected by *BubR1* than by *Mad2*. In contrast to our study, overexpression of BubR1 was reported to decrease patient survival in colorectal and bladder carcinoma (31,32). Further research is necessary to clarify the true role and determine the effects of BubR1 in gastric cancer.

In the present study, high *BubR1* mRNA expression was correlated with a low risk of relapse and improved survival in all patients, with the exception of patients with stage II and III disease. Since BubR1 is regulated by AURKB during chromosome alignment (33), we hypothesized that the combination of *BubR1* and *AURKB* expression might be of prognostic importance in gastric cancer. Indeed, stage II and III patients with high *BubR1* and/or high *AURKB* had an improved OS, and the combination of *BubR1* and *AURKB* was the only independent and significant prognostic factor for OS in these patients. These findings suggest that overexpression of *BubR1* and/or *AURKB* plays a crucial role in suppressing cancer growth.

In conclusion, we demonstrated the prognostic value of the combined assessment of *BubR1* and *AURKB* mRNA expression in gastric cancer patients. High *BubR1* and/or high *AURKB* expression identifies a highly favorable risk group among gastric cancer patients. Further studies are clearly required to verify these findings, establishing Mad2, BubR1 and AURKB as prognostic markers in gastric cancer, and functional analysis to clarify their role as tumor suppressors is needed.

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