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ORIGINAL ARTICLE

Prognostic impact of RING box protein-1 (RBX1) expression in gastric cancer

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Abstract
Background RING box protein-1 (RBX1) is an essential component of the E3 ubiquitin ligase Skp1/Cullin/RBX1/F-box protein complex. Although an altered expression of RBX1 has been reported in several human cancers, the role of RBX1 in gastric cancer remains unknown.
Methods We investigated the RBX1 expression in primary gastric cancer tissues from 145 patients by immunohistochemistry, and explored its clinical relevance and prognostic value. Furthermore, the effect of RBX1 expression on cancer cell proliferation was analyzed in vitro using a siRNA silencing technique.
Results The RBX1 expression was abundant in gastric cancer tissues. There was a significant difference in the expression level of RBX1 in terms of the tumor depth ($P = 0.008$), presence of distant metastasis ($P = 0.016$) and venous invasion ($P = 0.005$). The postoperative overall ($P < 0.001$) and relapse-free survival ($P < 0.001$) rates were significantly poorer in patients with RBX1-high tumors than in patients with RBX1-low tumors. There was a significant correlation of the RBX1 status with postoperative hematogenous recurrence ($P = 0.013$). Importantly, the RBX1 status was identified as an independent prognostic factor for gastric cancer ($P = 0.002$). Furthermore, RBX1 gene silencing significantly inhibited the proliferation of gastric cancer cells in vitro.
Conclusions The RBX1 expression has a significant prognostic value in gastric cancer. RBX1 might play an important role in regulating the proliferation of gastric cancer cells and promoting the development of postoperative recurrence. Our data provide a rationale for developing a novel therapy targeting RBX1 for gastric cancer.

Keywords RBX1 · E3 ubiquitin ligase · Gastric cancer

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Introduction

Gastric cancer is one of the most important causes of cancer-related death worldwide, and it remains a major public health concern in Japan [1, 2]. Although recent advances in the diagnosis, progress in surgical techniques and the development of new chemotherapeutic regimens have reduced gastric cancer deaths, a considerable number of patients, particularly those with advanced disease, still develop recurrence even after curative resection. Therefore, novel strategies against gastric cancer need to be developed and established to improve the patients' prognosis. Furthermore, the identification of markers to predict the outcomes of patients is crucial for appropriate treatment planning.

The ubiquitin-proteasome systems play a crucial role in controlling protein turnover and regulating a variety of signaling pathways and cellular processes [3]. SCF ubiquitin ligases, consisting of S-phase kinase-associated protein 1 (Skp1), Cullins, F-box proteins and a RING finger protein RBX1 [RING box protein-1/ROC1 (regulator of Cullins1)], are the largest multisubunit RING type E3 ubiquitin ligases and ubiquitinate a broad range of proteins, including cell cycle regulators, transcription factors, signal transducers and oncogenes/tumor suppressors [4–8]. Therefore, SCF dysfunction can cause or contribute to a variety of diseases, including cancer [8]. Indeed, the

upregulation or downregulation of components of SCF complexes, such as Skp2, Fbxw7, β-TrCP (β-transduction repeats-containing protein), Cullin1 and Cullin4, has been reported in various types of human cancers [8–12].

RBX1 was first isolated in yeast and was biochemically purified as an essential component of both the human and yeast SCF complexes, as well as of the von Hippel-Lindau tumor-suppressor complex [4–7, 13]. RBX1 acts as an adapter to catalyze the transfer of ubiquitin from the E2 ubiquitin-conjugated enzyme to substrates [4–7, 13]. RBX1 is ubiquitously expressed in human tissues, with the highest expression levels found in the heart, skeletal muscle, kidneys and placenta [14]. Although the overexpression of RBX1 has been indentified in a number of human primary cancer tissues, including carcinomas of the lung, breast, liver, colon, bladder and ovary, and in many cancer cell lines [15–17], the functions and role of RBX1 in tumors have not yet been fully elucidated. Moreover, the expression and clinical significance of RBX1 in gastric cancer remains unknown. In this study, we evaluated the RBX1 expression and attempted to clarify its clinical relevance and prognostic value in gastric cancer. Furthermore, the effect of RBX1 expression on cancer cell proliferation was analyzed in vitro using a siRNA silencing technique.

Patients and methods

Patients

We examined 145 patients with pathological stage IB-IV gastric cancer who underwent curative gastrectomy in the Department of Surgery, Nara Medical University, between January 2004 and December 2007. These selected patients had received neither chemotherapy nor radiotherapy before the operation. Tissue specimens, both cancerous and non-cancerous, were obtained from resected specimens and were then rapidly frozen at −80 °C for storage until use. For the immunohistochemical analyses, the remainder of each specimen was fixed in 10 % phosphate-buffered formalin and embedded in paraffin. Tumors were classified according to the TNM staging system [18]. The follow-up was until death or March 2013. The median follow-up for all patients was 60 months, with a range of 0.7–107.2 months. Written informed consent was obtained from all patients before the operation, according to our institutional guidelines.

Immunohistochemistry

The sections were stained using a DAKO EnVision system (Dako Cytomation, Kyoto, Japan), according to the manual provided by the manufacturer. As primary antibodies, a rabbit monoclonal anti-ROC1 antibody (ab133565, 1:250 dilution; Abcam, Tokyo, Japan) and a mouse monoclonal anti-human Ki67 antibody (M7240, 1:100 dilution; DAKO) were employed. Formalin-fixed, paraffin-embedded samples of primary tumor were cut into 5-μm sections, deparaffinized and rehydrated in a graded series of ethanol. Antigen retrieval was carried out by heating tissue sections using a Target Retrieval Solution at pH 6.0 (DAKO). To block endogenous peroxidase, sections were immersed in a 3 % (0.3 % for Ki67) solution of hydrogen peroxide in absolute methanol for 20 min (5 min for Ki67) at room temperature and washed three times in fresh PBS, with each wash lasting 5 min. Sections were then incubated with primary antibodies overnight at 4 °C. After three washes in PBS, the sections were incubated for 30 min with polymeric conjugate and washed three times with PBS. Reaction products were visualized with 3,3′-diaminobenzidine tetrahydrochloride, and the slides were counterstained with hematoxylin.

To evaluate the RBX1 and Ki67 expression, at least 1,000 tumor cells were scored in the invasive front of tumors at a magnification of ×400, and the percentage of tumor cells showing positive staining was calculated. A cutoff point for RBX1 was selected to give the optimal separation between a low and high risk in terms of the overall survival.

Gastric cancer cell lines

The human gastric cancer cell lines MKN45 (poorly differentiated adenocarcinoma) and MKN74 (moderately differentiated adenocarcinoma) were obtained from the RIKEN BioResource Center and then were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum.

Extraction of total mRNA and the real-time reverse transcriptase PCR analysis

Total RNA was isolated using the RNAspin Mini kit (GE Healthcare, UK, Ltd.), and the first-strand cDNA was synthesized from 1 μg RNA using a ReverTra Ace qPCR RT Kit (TOYOBO) according to the manufacturer's instructions. For the real-time reverse transcriptase PCR analysis, cDNA was amplified in TaqMan Fast Universal PCR Master Mix (2×; Applied Biosystems) with gene-specific primers and probes on the StepOnePlus Real-Time PCR System (Applied Biosystems), according to the manufacturer's instructions. The thermal cycling conditions were 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Real-time PCR experiments for each gene were carried out on three separate occasions. All primer/probe sets were purchased from Applied Biosystems. The expression level of the housekeeping gene, β_2-

microglobulin, was measured as an internal reference with a standard curve to determine the integrity of the template RNA for all of the specimens. The ratio of the mRNA level of each gene was calculated as follows: (absolute copy number of each gene)/(absolute copy number of β_2-microglobulin).

Preparation of cell lysates and a Western blot analysis

We resolved the cell lysates in SDS-polyacrylamide gels and transferred the proteins onto polyvinylidene difluoride membranes (Millipore, Ltd.). A rabbit monoclonal anti-ROC1 antibody (ab133565, 1:5000 dilution; Abcam) and a mouse monoclonal anti-actin antibody (C-2, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were employed. The membranes were incubated with the indicated primary antibodies overnight at 4 °C, and then were incubated with horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology). We detected the peroxidase activity on X-ray films using an enhanced chemiluminescence detection system.

Transfection of the siRNA

For our transfection analyses, MKN45 and MKN74 cells were transfected with either control RNA (QIAGEN) or 40 nmol/l of siRNA against RBX1. Transfections were carried out using the Lipofectamine system (Invitrogen) in accordance with the manufacturer's protocol when cells achieved about 30 % confluence. The human RBX1 siRNA duplexes, generated with 3′-dTdT overhangs and prepared by QIAGEN, were chosen to target the following DNA sequence: 5′-AAGAAGCGCTTTGAAGTGAAA-3′.

Cell viability assay

Cell viability was determined using the Cell-Titer 96 Aqueous One Solution cell proliferation assay kit, according to the manufacturer's instruction manual (Promega Corp., Madison, WI, USA). Briefly, aliquots of 8×10^3 of MKN45 cells and 3×10^3 of MKN74 cells per well were cultured in 96-well plates. After 24 h, the cells were transfected with control RNA or RBX1 siRNA. Cell-titer 96 Aqueous One Solution was added to each well 72 h after transfection, and cells were incubated for an additional 2 h. The absorbance at 490 nm in each well was recorded with a 96-well plate reader. Each experiment was performed at least three times.

Statistical analysis

Continuous variables were expressed as the means and standard deviations, and the means were compared using Student's t test or a one-way ANOVA. Categorical variables were presented as numbers and percentages, and groups were compared using the chi-square test or Fisher's exact test. The overall survival was defined as the time from the operation until death. The relapse-free survival was defined as the time from the operation to the relapse of the disease. The survival curves were calculated by the Kaplan–Meier method. Differences between the curves were analyzed by the log-rank test. The univariate and multivariate hazard ratios (HRs) were calculated using the Cox proportional hazard model. All significant variables in the univariate analysis were entered into a multivariate analysis. The correlation between the RBX1 and Ki67 expression was analyzed by a Pearson correlation analysis. A value of $P < 0.05$ was considered to be significant, and confidence intervals (CI) were calculated at the 95 % level. The statistical analyses were performed using the SPSS® software program, version 19.0 (SPSS, Chicago, IL, USA).

Results

RBX1 expression in human gastric cancer

We first compared the relative expression levels of RBX1 between gastric cancer tissues and non-cancer tissues using available frozen tissues. Real-time PCR showed that gastric cancer tissues expressed much higher levels of RBX1 mRNA than non-cancer tissues ($P = 0.004$; Fig. 1a). When evaluating individual patients, the RBX1 expression level of cancer tissues was higher than that of non-cancer tissues in 13 (86.7 %) out of 15 patients (Fig. 1b).

We next examined the RBX1 protein expression in gastric cancer tissue specimens by immunohistochemistry. In all gastric cancer tissue specimens, positive staining for RBX1 was seen in the nuclei of cancer cells. Positive staining for RBX1 was also seen in the cytoplasm of some cancer cells. Overall, the mean percentage of RBX1-positive cells in gastric cancer tissues was 51.6 % (standard deviation 23.7 %). In non-cancer tissues, some mononuclear cells were also positive for RBX1. These data suggest that RBX1 might therefore play some roles in human gastric cancer.

Clinical significance of the RBX1 expression in human gastric cancer

We further investigated the clinical relevance of the RBX1 expression in gastric cancer. The relationship between the clinicopathological findings and the RBX1 expression is shown in Table 1. The rate of RBX1-positive tumor cells in differentiated cancer was significantly higher than that in undifferentiated cancer ($P < 0.001$). There was a significant difference in the positive rate of RBX1 in terms of the tumor depth ($P = 0.008$). The tumors with distant metastasis ($P = 0.016$) and venous invasion ($P = 0.005$) also had a significantly

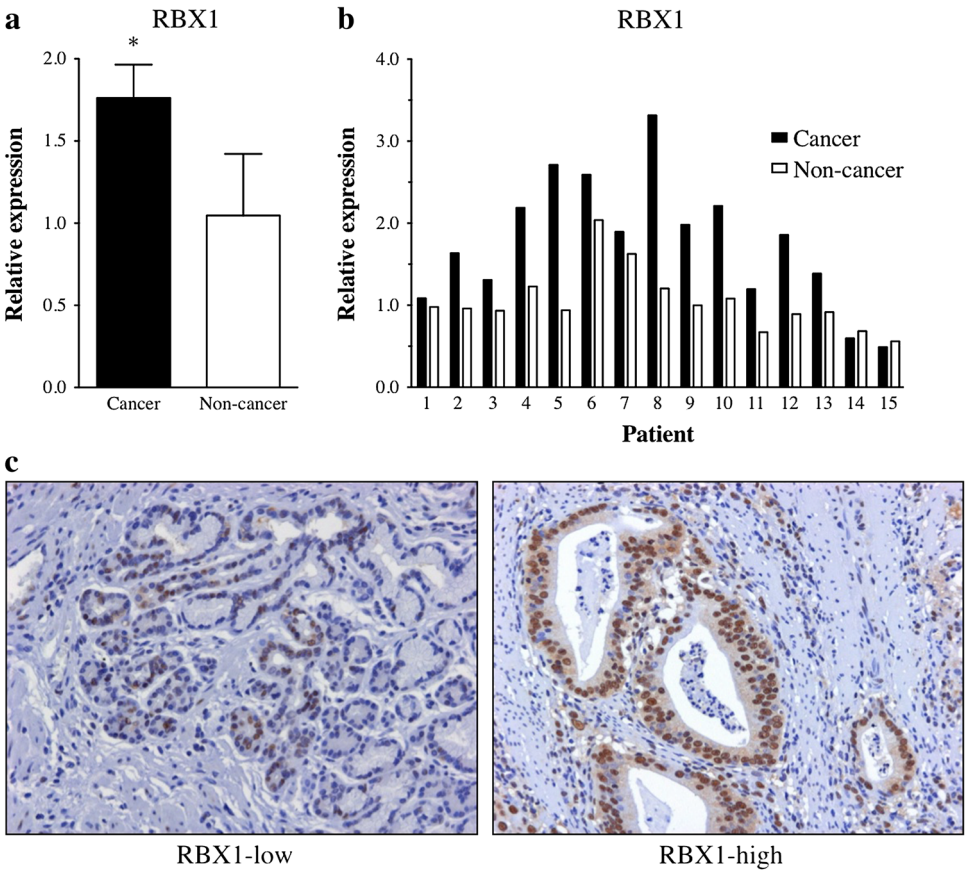

Fig. 1 The RBX1 expression in gastric cancer. **a** The RBX1 expression of cancer tissue specimens ($n = 15$) was significantly higher than that of non-cancer tissue ($n = 15$; $P = 0.004$). **b** In 86.7 % of the gastric cancer patients, the RBX1 expression of cancer tissues was higher than that of non-cancer tissues. **c** Representative cases of low and high expression of RBX1. Original magnification, ×200

higher mean percentage of RBX1-positive tumor cells than the tumors without such factors. These data suggested that RBX1 might be involved in the progression of gastric cancer.

Association of the RBX1 expression with the postoperative prognosis

In order to investigate the influence of the RBX1 expression on the postoperative prognosis of gastric cancer, all specimens were classified into two groups according to the percentage of RBX1-positive tumor cells. The hazard ratio for high RBX1 for the overall survival was highest when the cutoff value of the RBX1 expression was 48 % (HR 3.599). Therefore, the cutoff value of the RBX1 expression was set at 48 %. Then, 89 patients (61.4 %) with a RBX1-positive rate of 48 % or higher and 56 patients (38.6 %) with a RBX1-positive rate less than 48 % were classified into RBX1-high and RBX1-low groups, respectively (Fig. 1c). The 5-year overall survival rate was significantly lower in the RBX1-high group than in the RBX1-low group (54.9 versus 85.1 %, $P < 0.001$; Fig. 2a).

Impact of the RBX1 status on postoperative recurrence

We further analyzed the impact of the RBX1 status on postoperative recurrence. At the time of analysis, 39 patients (26.9 %) had postoperative recurrence. The site of the first relapse was the peritoneum in 22 patients, hematogenous in 13, lymph node in 6 and local in 1. Overall, the rate of recurrence was higher in the RBX1-high group than in the RBX1-low group (Table 2). Hematogenous recurrence was significantly more common in the RBX1-high group than in the RBX1-low group ($P = 0.013$; Table 2). Furthermore, the 5-year relapse-free survival rate was significantly lower in the RBX1-high group than in the RBX1-low group (52.5 versus 83.7 %, $P < 0.001$; Fig. 2b).

Prognostic value of the RBX1 status in gastric cancer

In the univariate analysis of the overall survival, the HR for high RBX1 expression was 3.599 (95 % CI 1.741–7.44, $P = 0.001$). The other factors that significantly correlated with the patients' overall survival were the tumor size, tumor depth, lymph node metastasis, distant metastasis and venous invasion. The multivariate analysis demonstrated that the RBX1 status and the tumor size, lymph node metastasis and distant metastasis were independent prognostic factors for the overall survival ($P = 0.002$; Table 3). Taken together, these data indicate that RBX1 might be a molecular prognostic marker for gastric cancer.

Table 1 Association between the clinicopathological characteristics and the RBX1 expression

Variables	*n*	Percentage of RBX1-positive tumor cells	*P* value
Gender			
Male	108	53.3 ± 23	0.149[a]
Female	37	46.8 ± 25.3	
Age (years)			
<67	67	45.8 ± 23.5	0.005[a]
≥67	78	56.7 ± 22.8	
Histology			
Differentiated	71	60 ± 21.7	<0.001[a]
Undifferentiated	74	43.6 ± 22.8	
Tumor size (mm)			
<50	89	49.2 ± 24	0.114[a]
≥50	56	55.6 ± 22.7	
Tumor depth			
T1	19	38 ± 25.9	0.008[b]
T2	50	52.8 ± 23.1	
T3	42	49.3 ± 23.5	
T4	34	60.4 ± 20.2	
Lymph node metastasis			
Negative	51	51.7 ± 21.8	0.98[a]
Positive	94	51.6 ± 24.7	
Distant metastasis			
Absent	137	50.8 ± 23.8	0.016[a]
Present	8	67 ± 14.6	
Pathological stage			
IB	43	50.9 ± 22	0.115[b]
II	51	47.2 ± 25.7	
III	43	54.8 ± 23.1	
IV	8	67 ± 14.6	
Lymphatic invasion			
Negative	31	48.2 ± 23.7	0.362[a]
Positive	114	52.6 ± 23.7	
Venous invasion			
Negative	91	47.6 ± 24.4	0.005[a]
Positive	54	58.5 ± 20.9	

Values are expressed as the means and standard deviations

RBX1 RING box protein-1

[a] Indicates the value obtained by Student's *t* test

[b] Indicates the value from a one-way ANOVA

Correlation between the RBX1 and Ki67 expression in human gastric cancer

We further investigated the involvement of RBX1 in gastric cancer cell proliferation, since RBX1 has been suggested to be required for cancer cell proliferation [15, 16, 19]. Overall, the mean percentage of Ki67-positive tumor cells was 21.9 % (standard deviation 12.7 %). The Ki67-positive rate was significantly higher in RBX1-high tumors than in RBX1-low tumors (25.8 ± 12.8 versus 15.7 ± 9.8 %, $P < 0.001$; Fig. 3a). Furthermore, the RBX1 expression level was significantly correlated with the Ki67 expression level ($P < 0.001$, $r = 0.409$; Fig. 3b).

RBX1 gene silencing inhibits the proliferation of gastric cancer cells

We finally performed in vitro experiments to examine the direct relationship between the RBX1 expression and cancer cell proliferation. To this end, we used human gastric cancer cell lines MKN45 and MKN74 and examined the effects of RBX1 downregulation using a siRNA knockdown approach. At 72 h post-transfection, RBX1 siRNA significantly reduced both the mRNA and protein expression levels of RBX1 compared with the control siRNA in both cell lines (Fig. 4a, b).

We then examined the role of RBX1 in the regulation of cancer cell proliferation by the MTS assay. As shown in Fig. 4c, the cell proliferation was significantly suppressed by RBX1 gene silencing for up to 72 h in both MKN45 and MKN74 cells compared to cells transfected with the control siRNA. Therefore, the data suggested that RBX1 plays an important role in regulating the proliferation of gastric cancer cells.

Discussion

In this study, we investigated the expression of the RING finger protein RBX1 and addressed its clinical significance and prognostic value in gastric cancer. A number of previous studies have reported altered expression levels of components of SCF complexes and their prognostic impact in several human cancers [8, 9, 11, 12]. In gastric cancer, high expression of Skp2 has been reported to be associated with a worse prognosis [9]. The high expression of Cullin1 has also been associated with a higher frequency of lymph node metastasis and worse prognosis [11]. Furthermore, the Cullin1 status has been identified as an independent prognostic marker for gastric cancer. To date, however, the role of RBX1 in human gastric cancer remains largely unknown. Our study showed that a higher expression of RBX1 correlated significantly with a worse postoperative overall survival. Importantly, the RBX1 status was an independent predictor for the postoperative survival of gastric cancer patients, independent of the TNM classification. Therefore, data suggest that RBX1 could be a novel prognostic biomarker for gastric cancer.

Dysfunction of SCF complexes has been associated with tumorigenesis [8]. Latres et al. [20] showed that Skp2 cooperated with activated N-Ras during the tumorigenesis

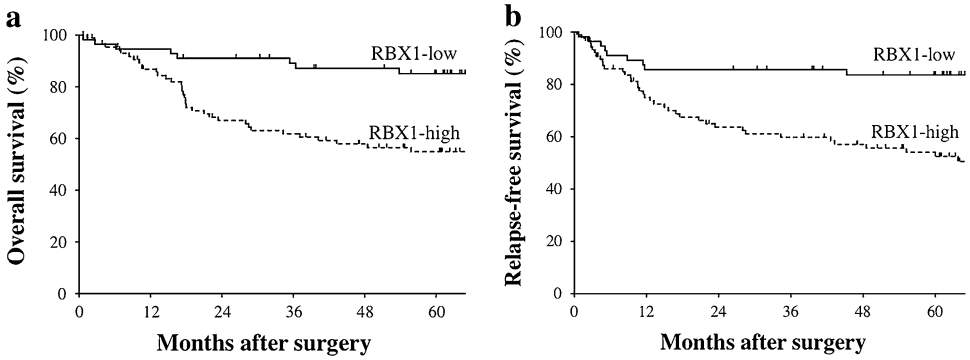

Fig. 2 The postoperative survival was significantly poorer in patients with RBX1-high tumors than in those with RBX1-low tumors. **a** The overall survival ($P < 0.001$). **b** The relapse-free survival ($P < 0.001$)

Table 2 Impact of the RBX1 status on the postoperative recurrence

Site	RBX1-low ($n = 56$, %)	RBX1-high ($n = 89$, %)	OR	95 % CI	P value
All recurrence	9 (16.1)	30 (33.7)	2.655	1.149–6.137	0.02[a]
Peritoneum	7 (12.5)	15 (16.9)	1.419	0.539–3.732	0.477[a]
Hematogenous	1 (1.8)	12 (13.5)	8.571	1.083–67.868	0.013[b]
Lymph node	2 (3.6)	4 (4.5)	1.271	0.225–7.176	0.573[b]

Some patients had a first recurrence at more than one site

RBX1 RING box protein-1, *OR* odds ratio, *CI* confidence interval

[a] Indicates data obtained by the Chi-square test

[b] Indicates data obtained by Fisher's exact test

of T-cell lymphoma in a transgenic mouse model. While approximately 35 % of the transgenic mice expressing activated N-Ras developed T-cell lymphoma, 75 % of the transgenic mice expressing both activated N-Ras and Skp2 developed T-cell lymphoma. In the present study, we confirmed the overexpression of RBX1 at both the mRNA and protein levels in human gastric cancer tissues. We then examined the correlation of the RBX1 expression with the clinicopathological factors in human gastric cancer tissue specimens and found that the expression level of RBX1 was significantly higher in differentiated tumors than in undifferentiated tumors. This finding was in conflict with the data reported by Bai et al. [11] who observed that there was no significant association between the expression level of Cullin1 and the differentiation of gastric cancer. These data suggest that RBX1 may be implicated in the pathogenesis of gastric cancer and that the contributions of RBX1 may differ between differentiated and undifferentiated gastric cancer. However, the precise mechanism(s) underlying the difference in the RBX1 expression levels between differentiated and undifferentiated gastric cancer remain unclear. Therefore, further investigations are needed.

There have been limited studies on RBX1 in human cancer tissue samples. Yang et al. [16] found the overexpression of RBX1 in 151 hepatocellular carcinomas, and they noted that the RBX1 status correlated positively with the tumor size and pathological differentiation grade, and

Table 3 The results of the multivariate analysis for survival

Variables		HR	95 % CI	P value[a]
Tumor size (mm)	≥50/<50	1.975	1.074–3.634	0.029
Tumor depth	T3, T4/T1, T2	1.248	0.65–2.394	0.506
Lymph node metastasis	Positive/ negative	2.614	1.175–5.814	0.018
Distant metastasis	Present/ absent	3.68	1.482–9.139	0.005
Venous invasion	Positive/ negative	1.373	0.741–2.543	0.314
RBX1 expression	High/low	3.272	1.554–6.886	0.002

HR hazard ratio, *CI* confidence interval, *RBX1* RING box protein-1

[a] Indicates data obtained from the Cox proportional hazard model

negatively with the survival rate of patients. Wang et al. [17] investigated the RBX1 expression in 70 non-muscle-invasive bladder transitional cell carcinoma (NMIBC) patients and found that high RBX1 expression was significantly associated with a high tumor grade, advanced clinical stage, and shorter recurrence-free and progression-free survival. Furthermore, the authors identified the RBX1 status as an independent prognostic marker for the recurrence and progression of NMIBC. On the other hand, a previous study showed a remarkably high frequency of both genetic disruption and gene expression changes for the genes encoding E3 ubiquitin ligase protein components,

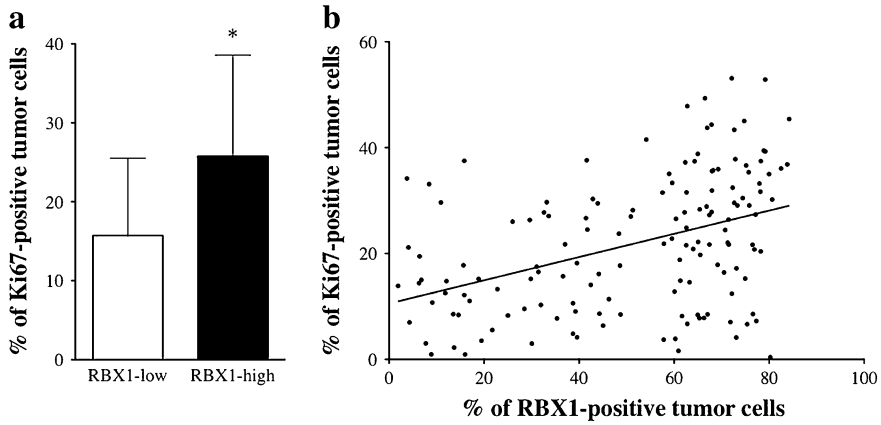

Fig. 3 The relationship between the RBX1 and Ki67 expression in gastric cancer. **a** The RBX1-high tumors had a significantly higher percentage of Ki67-positive tumor cells than the RBX1-low tumors ($P < 0.001$). **b** There was a significant correlation between the RBX1 and Ki67 expression levels ($P < 0.001$, $r = 0.409$)

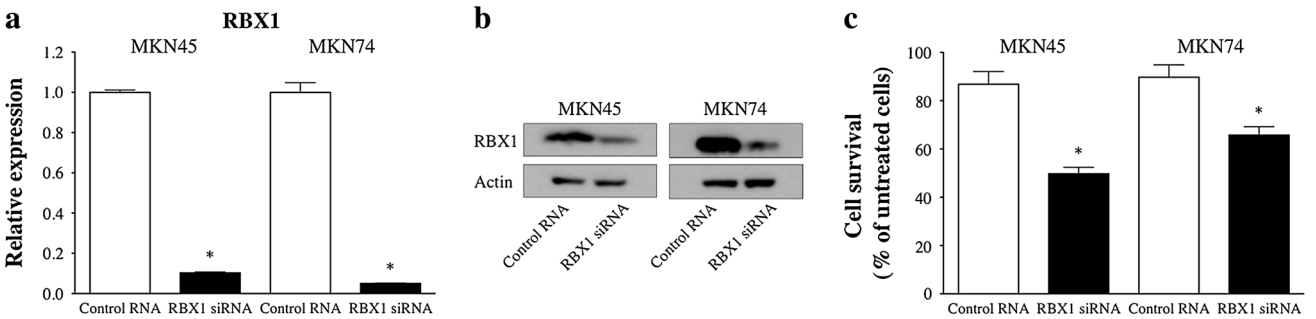

Fig. 4 The downregulation of RBX1 by siRNA inhibits the proliferation of human gastric cancer cells. **a** MKN45 and MKN74 cells were transfected with control RNA or RBX1 siRNA. The RBX1 expression was evaluated by quantitative real-time PCR. The expression of RBX1 mRNA was strongly reduced for up to 72 h in both cell lines following transfection of the RBX1 siRNA. $n = 3$ in each group. **b** The total protein lysates were extracted from the treated cells and subjected to an immunoblotting analysis. The protein expression of RBX1 was also effectively suppressed in both cell lines. **c** The cell proliferation was significantly inhibited in cells treated with RBX1 siRNA compared to those treated with the control siRNA, as determined by the MTS assay after 72 h of incubation ($n = 10$ for each group). *$P < 0.001$

including RBX1, in lung cancer [21]. Another study found a significantly lower expression level of RBX1 in melanoma compared to melanocytic nevi [22]. Thus, the roles of RBX1 in human cancers seem complex. In this study, we found the RBX1 expression level to be significantly associated with the tumor depth, distant metastasis and venous invasion. These data suggested that RBX1 might be involved in promoting the invasion and metastasis of gastric cancer.

In addition, our study clearly demonstrated that the tumors with high RBX1 expression were associated with a higher risk of gastric cancer recurrence. The relapse-free survival rate was significantly poorer in patients with RBX1-high tumors than in those with RBX1-low tumors. Furthermore, there was a significant correlation between the RBX1 status and postoperative hematogenous recurrence. This higher frequency of hematogenous recurrence in RBX1-high tumors might be partly explained by the significant correlation between the RBX1 expression level and venous invasion [23]. On the other hand, the RBX1 status did not correlate with peritoneal or lymph node recurrence. Taken together, these data suggest that RBX1 may affect the development of postoperative hematogenous recurrence, and our data emphasize that the detection of the RBX1 expression might be helpful for the prediction of gastric cancer recurrence.

In this study, we also demonstrated that the RBX1 expression level was associated with the proliferation of gastric cancer. RBX1 has been recently suggested to be involved in the regulation of cancer cell growth and survival. A short hairpin RNA-mediated functional genetic screen identified RBX1 as a growth essential gene in a number of human cancer cell lines [19]. In this study, we evaluated the proliferative activity by Ki67 staining, since the Ki67 nuclear antigen is a marker of proliferation and is a good indicator of the proliferation and differentiation capacity of gastric cancer cells [24]. The RBX1-high tumors had a significantly higher percentage of Ki67-positive tumor cells than the RBX1-low tumors. Furthermore, the expression level of RBX1 positively correlated with that of Ki67. In addition, we examined the relationship between the RBX1 expression and the proliferation of gastric cancer cells in vitro by using a siRNA method and found that the cell proliferation was significantly inhibited

by RBX1 gene silencing. These data suggest that RBX1 might promote the growth of gastric cancer cells by increasing cell proliferation. However, further studies are required to clarify the molecular mechanism underlying the role of RBX1 in gastric cancer progression.

Given the overexpression and prognostic value of molecules involved in the SCF complex, they seem to represent an attractive target for cancer treatment. More recently, a small molecule inhibitor of NEDD8-activating enzyme, MLN4924, which blocks Cullin neddylation, was discovered [25]. MLN4924 inactivates SCF, leading to the accumulation of SCF substrates and the suppression of cancer cell growth [25]. MLN4924 is currently under investigation in clinical trials [26]. Jia et al. [15] reported that RBX1 gene silencing remarkably inhibited tumor cell growth by inducing G_2/M arrest, apoptosis and senescence. Another study also showed that RBX1 gene silencing suppressed the growth of liver cancer cells both in vitro and in vivo by inducing autophagy and p21-dependent cell senescence [16]. Taken together, these studies suggest that RBX1 might be a promising therapeutic target for cancer.

In conclusion, we have shown for the first time that the RBX1 expression has a significant prognostic value in gastric cancer, independent of the conventional TNM classification. Furthermore, our study has suggested that RBX1 might play an important role in regulating the proliferation of gastric cancer cells and promoting the development of postoperative recurrence. Our data may provide a rationale for developing a novel therapy targeting RBX1 for gastric cancer.

Conflict of interest None of the authors has any financial conflicts to disclose in association with this study.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55:74–108.
2. Nashimoto A, Akazawa K, Isobe Y, Miyashiro I, Katai H, Kodera Y, et al. Gastric cancer treated in 2002 in Japan: 2009 annual report of the JGCA nationwide registry. Gastric Cancer. 2013;16:1–27.
3. Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem. 1998;67:425–79.
4. Skowyra D, Koepp DM, Kamura T, Conrad MN, Conaway RC, Conaway JW, et al. Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and Rbx1. Science. 1999;284:662–5.
5. Ohta T, Michel JJ, Schottelius AJ, Xiong Y. ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. Mol Cell. 1999;3:535–41.
6. Tan P, Fuchs SY, Chen A, Wu K, Gomez C, Ronai Z, et al. Recruitment of a ROC1-CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of I kappa B alpha. Mol Cell. 1999;3:527–33.
7. Seol JH, Feldman RM, Zachariae W, Shevchenko A, Correll CC, Lyapina S, et al. Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev. 1999;13:1614–26.
8. Frescas D, Pagano M. Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. Nat Rev Cancer. 2008;8:438–49.
9. Masuda TA, Inoue H, Sonoda H, Mine S, Yoshikawa Y, Nakayama K, et al. Clinical and biological significance of S-phase kinase-associated protein 2 (Skp2) gene expression in gastric carcinoma: modulation of malignant phenotype by Skp2 overexpression, possibly via p27 proteolysis. Cancer Res. 2002;62:3819–25.
10. Welcker M, Clurman BE. FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. Nat Rev Cancer. 2008;8:83–93.
11. Bai J, Zhou Y, Chen G, Zeng J, Ding J, Tan Y, et al. Overexpression of Cullin1 is associated with poor prognosis of patients with gastric cancer. Hum Pathol. 2011;42:375–83.
12. Birner P, Schoppmann A, Schindl M, Dinhof C, Jesch B, Berghoff AS, et al. Human homologue for Caenorhabditis elegans CUL-4 protein overexpression is associated with malignant potential of epithelial ovarian tumours and poor outcome in carcinoma. J Clin Pathol. 2012;65:507–11.
13. Kamura T, Koepp DM, Conrad MN, Skowyra D, Moreland RJ, Iliopoulos O, et al. Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science. 1999;284:657–61.
14. Swaroop M, Gosink M, Sun Y. SAG/ROC2/Rbx2/Hrt2, a component of SCF E3 ubiquitin ligase: genomic structure, a splicing variant, and two family pseudogenes. DNA Cell Biol. 2001;20:425–34.
15. Jia L, Soengas MS, Sun Y. ROC1/RBX1 E3 ubiquitin ligase silencing suppresses tumor cell growth via sequential induction of G2-M arrest, apoptosis, and senescence. Cancer Res. 2009;69:4974–82.
16. Yang D, Li L, Liu H, Wu L, Luo Z, Li H, et al. Induction of autophagy and senescence by knockdown of ROC1 E3 ubiquitin ligase to suppress the growth of liver cancer cells. Cell Death Differ. 2013;20:235–47.
17. Wang W, Qiu J, Liu Z, Zeng Y, Fan J, Liu Y, et al. Overexpression of RING box protein-1 (RBX1) associated with poor prognosis of non-muscle-invasive bladder transitional cell carcinoma. J Surg Oncol. 2013;107:758–61.
18. Sobin L, Gospodarowicz M, Wittekind C, editors. UICC-TNM classification of malignant tumors. 7th ed. New York: Wiley-Blackwell; 2010.
19. Schlabach MR, Luo J, Solimini NL, Hu G, Xu Q, Li MZ, et al. Cancer proliferation gene discovery through functional genomics. Science. 2008;319:620–4.
20. Latres E, Chiarle R, Schulman BA, Pavletich NP, Pellicer A, Inghirami G, et al. Role of the F-box protein Skp2 in lymphomagenesis. Proc Natl Acad Sci USA. 2001;98:2515–20.
21. Thu KL, Pikor LA, Chari R, Wilson IM, Macaulay CE, English JC, et al. Genetic disruption of KEAP1/CUL3 E3 ubiquitin ligase complex components is a key mechanism of NF-kappaB pathway activation in lung cancer. J Thorac Oncol. 2011;6:1521–9.
22. Nai G, Marques M. Role of ROC1 protein in the control of cyclin D1 protein expression in skin melanomas. Pathol Res Pract. 2011;207:174–81.
23. Moriguchi S, Maehara Y, Korenaga D, Sugimachi K, Nose Y. Risk factors which predict pattern of recurrence after curative surgery for patients with advanced gastric cancer. Surg Oncol. 1992;1:341–6.
24. Wang L, Zheng L, Wang SY, Zhu TF, Zhu HG. Clonal analysis of gastric carcinoma and precancerous lesions and its relation to Ki-67 protein expression. Neoplasma. 2009;56:48–55.

25. Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, et al. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature. 2009;458:732–6.

26. Soucy TA, Dick LR, Smith PG, Milhollen MA, Brownell JE. The NEDD8 Conjugation Pathway and Its Relevance in Cancer Biology and Therapy. Genes Cancer. 2010;1:708–16.