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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

High RAD18 Expression is Associated with Disease Progression and Poor Prognosis in Patients with Gastric Cancer

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ABSTRACT

Background. RAD18 plays an important role in DNA damage repair by inducing monoubiquitinated PCNA (mUB-PCNA) in both cancer and normal tissues. Previous studies have not determined the significance of RAD18 expression in clinical gastric cancer (GC) samples. Thus, this study aimed to clarify the expression and functional significance of RAD18 in GC.

Methods. Overall, 96 resected GC samples were subjected to an immunohistochemical analysis of RAD18. GC cell lines were also subjected to functional RNA interference analyses of RAD18.

Results. RAD18 expression was predominantly nuclear and was observed at higher levels in GC tissues than in normal tissues. In GC tissues, strong RAD18 expression was associated with progression of lymph node metastasis (p = 0.0001), lymphatic invasion (p = 0.0255), venous invasion ($p < 0.0001$), recurrence (p = 0.028), and disease stage (p = 0.0253). Moreover, GC patients with high tumor RAD18 expression had shorter overall survival (p = 0.0061) and recurrence-free survival durations (p = 0.035) than those with low tumor RAD18 expression. RAD18 knockdown inhibited GC proliferation and invasiveness and increased chemosensitivity by suppressing mUB-PCNA.

Conclusions. RAD18 expression may be a useful marker of progression and poor prognosis of GC. Moreover, therapeutic strategies that target RAD18 might be a novel chemosensitizer to eradicate the refractory GC.

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Gastric cancer (GC) is the fifth most common cancer and a leading cause of cancer-related deaths worldwide.[1] Patients with operable GC should undergo surgical resection as a curative treatment. Postoperative chemotherapy can improve patient survival and prevent disease recurrence.[2] Accordingly, many patients with locally advanced or metastatic GC have been treated with systemic chemotherapy. However, chemotherapy-refractory GC is generally associated with a poor prognosis.[3] Therefore, new biomarkers and therapeutic targets need to be identified to improve treatment efficacies and prognoses in patients with refractory GC.

RAD18, a DNA repair protein, plays a critical role in initiating DNA damage repair signaling. RAD18 acts as an E3 ligase to monoubiquitinate proliferating cell nuclear antigen (PCNA) to yield monoubiquitinated PCNA (mUB-PCNA), an important regulator of DNA repair. Therefore, the function of RAD18 in both cancer and non-cancerous cells was activated in DNA damage response. On the other

hand, the expression of RAD18 in cancer cells was reported to be regulated by not only DNA damage response but also long non-coding RNA and de-ubiquitylating enzyme.[4,5]

RAD18 suppression was clearly associated with an increase in DNA damage induced by ultraviolet (UV) irradiation and the anticancer drug CDDP mediated by mUB-PCNA inhibition.[4] Moreover, high RAD18 expression levels in esophageal cancer tissues were associated with tumor progression and a poor prognosis.[6] These studies confirmed the role of RAD18 as a fundamental DNA damage repair gene and potential target for the diagnosis and treatment of cancers; however, the exact significance of RAD18 in tumor progression and therapeutic resistance has not yet been elucidated in patients with GC.

The present study aimed to clarify the significance of RAD18 expression in GC patients by subjecting 96 clinical GC samples to immunohistochemical (IHC) testing. We further aimed to determine the functions of RAD18 in GC through an analysis of the effects of RAD18 suppression on the proliferation and invasion capacities and chemosensitivity of GC cell lines.

MATERIALS AND METHODS

Clinical Samples and Cell Lines

Surgical specimens were obtained from 96 patients with GC (79 men and 17 women) who underwent potentially curative surgery at the Department of General Surgical Science, Gunma University, between 1999 and 2013. None of the patients had received preoperative irradiation or chemotherapy. This study conformed to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board for Clinical Research at Gunma University Hospital (Maebashi, Gunma, Japan; approval number HS2019-043). Patient consent was obtained using the opt-out method. Curative surgery was defined as a case with a lack of evidence of residual tumor and microscopically tumor-free resection margins (R0). The pathological features of the specimens were classified based on the 14th edition of the Japanese Classification of Gastric Carcinoma outlined by the Japanese Gastric Cancer Association.

The human GC cell lines MKN7, MKN74, MKN45, and GCIY were purchased from the JCRB Cell Bank (Osaka, Japan) and RIKEN BRC (Tokyo, Japan). All cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Gibco, NY, USA). The cultured cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

Immunohistochemical (IHC) Analysis

Paraffin-embedded GC specimens were cut into 2-μm-thick sections that were mounted on glass slides. All sections were incubated at 60 °C for 60 min, deparaffinized in xylenes, rehydrated, and incubated with fresh 0.3% hydrogen peroxide in 100% methanol for 30 min to block endogenous peroxidase activity. After rehydration through a graded series of ethanol treatments, antigen retrieval was performed using Immunosaver (Nishin EM, Tokyo, Japan) at 98–100 °C for 45 min. The sections were then passively cooled to room temperature and incubated in protein block serum-free reagent (DAKO, Carpinteria, CA, USA) for 30 min. Next, the specimens were incubated with a rabbit polyclonal anti-RAD18 antibody (Abcam, Cambridge, UK) at a 1:100 dilution in PBS containing 0.1% bovine serum albumin at 4 °C for 24 h. Primary antibody staining was visualized using the Histofine Simple Stain MAX-PO (Multi) Kit (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. The chromogen 3,3-diaminobenzidine tetrahydrochloride was applied as a 0.02% solution in 50 mM ammonium acetate-citrate acid buffer (pH 6.0) containing 0.005% hydrogen peroxide. The sections were lightly counterstained with hematoxylin and mounted. Lymphoid tissues were used as a positive control of RAD18 staining. Negative controls were incubated without the primary antibody, and no detectable staining was evident. IHC slides were evaluated by three experienced researchers who had no knowledge of the clinical data. The following staining scores for each sample were set as the average of the evaluation. Immunohistochemistry was scored based on the intensity of cells with nuclear RAD18 staining. The intensity was scored as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining (Fig. 1). A staining score ≥ 1.0 was defined as the high-expression group, while a score < 1.0 was defined as the low-expression group.

Small-Interfering RNA Transfection

RAD18-specific small-interfering RNA (siRNA) oligos (RAD18 siRNA1: GCCAAGGAAAGAUGCUAAtt; RAD18 siRNA2: ACAGUGAAGUGCAGACAUUtt) and non-targeting control siRNA oligos (NC siRNA) were purchased from GeneDesign Corporation (Osaka, Japan). The target cell lines MKN45 and GCIY were subjected to RNA interference using an in vitro electroporation protocol. The cells were suspended for a short period in serum-free Opti-MEM I (Life Technologies, Carlsbad, CA, USA) at a density of 1×10^7 cells/mL, after which siRNA was

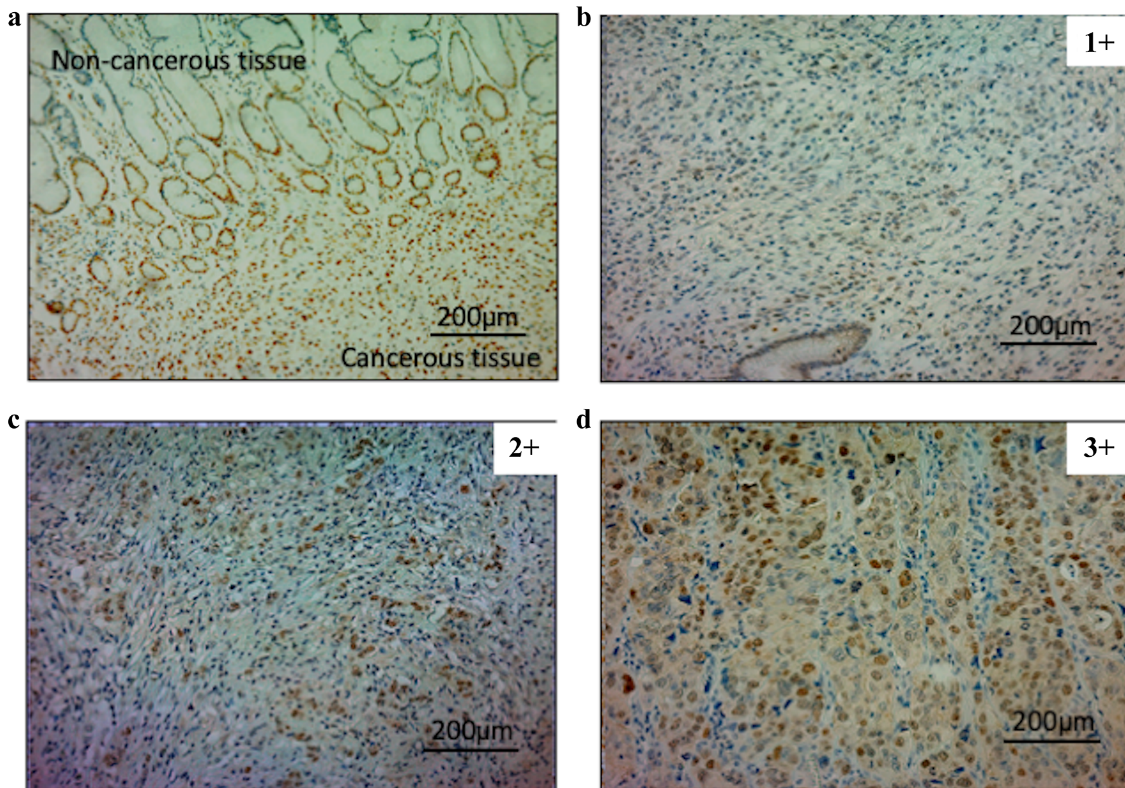

FIG. 1 Immunohistochemical staining of RAD18 in clinical GC samples. **a** Representative immunohistochemical staining of RAD18 in cancerous areas of GC tissues and non-cancerous gastric mucosa (normal; original magnification ×200). Stronger RAD18 expression was observed in GC tissues than in normal gastric mucosa. **b** Representative section of a GC tissue with low RAD18 expression. **c** Representative section of a GC tissue with moderate RAD18 expression. **d** Representative section of a GC tissue with high RAD18 expression. *GC* gastric cancer

added to the cell suspension at a concentration of 10 nM. Subsequently, 100 μL of the cell suspension was transferred to a 2-mm gap cuvette electrode and subjected to electroporation using an electroporator (CUY21EDIT II; BEX Co., Japan), as previously described.[7]

Protein Extraction and Western Blot Analysis

Total proteins (10 μg) were electrophoresed on a polyacrylamide gel and electroblotted to a nitrocellulose membrane at 300 mA for 90 min. Western blotting was used to confirm the expression of target proteins detected using the following reagents: anti-RAD18 rabbit polyclonal antibody (1:1000; Abcam), PCNA (D3H8P) XP rabbit mAb (1:1000; Cell Signaling Technology, Danvers, MA, USA), ubiquityl-PCNA (Lys164) [D5C7P] rabbit mAb (1:1000; Cell Signaling Technology), anti-gamma H2A.X (phospho S139) [1:1000; Abcam], and β-actin mouse monoclonal antibody (1:1000; Cell Signaling Technology). β-actin expression was used as a protein loading control. The blots were detected using the ECL Western Blot Analysis Detection System and an Image Quant LAS 4000 machine (GE Healthcare Life Sciences, Marlborough, MA, USA).

Cell Proliferation Assay

The cells were plated in 96-well plates in a volume of 100 μL and approximate density of 300 cells/well. A Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was used to quantify cell viability. Ten microliters of cell-counting solution were added to each well, after which the plates were incubated at 37 °C for 2 h. The cell proliferation rate was then determined by measuring the absorbance in each well at 450 nm using a microtiter plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Invasion Assay

The invasiveness of MKN45 and GCIY cells was analyzed using Matrigel-coated transwell chambers (Corning, New York, NY, USA). The lower chambers were placed in 24-well plates and filled with RPMI 1640 containing 10% FBS. GC cells (density: 1.0×10^5) were seeded in serum-

free media in the upper chambers of the Matrigel-coated chambers. After a 48-h incubation, the chambers were removed and washed with PBS. The cells were then fixed in methanol and stained with Diff-Quick stain (Sysmex, Kobe, Japan). The membranes were cut and observed using bright-field microscopy at a magnification of 100×.

Cisplatin Sensitivity Assay

The cisplatin sensitivity of cells treated with NC siRNA or RAD18 siRNA was measured. The cells were plated in 96-well plates in 100 μL of medium at a density of 3000 cells/well. After a 24-h incubation, the cells were treated with various concentrations of cisplatin (Nichi-Iko Pharmacological Co., Toyama, Japan) for 48 h. Cell viability was assessed using CCK-8. The absorbance data were used to calculate the half maximal inhibitory concentration (IC_{50}).

Statistical Analysis

Student's *t* test and the Chi square test were used to identify statistically significant differences between the two groups. Kaplan–Meier curves of overall and recurrence-free survival were generated from clinical data. Statistical significance was determined using the log-rank test. Univariate and multivariate survival analyses were performed using the Cox proportional hazards model. A p value < 0.05 was considered to indicate statistical significance. All statistical analyses were performed using JMP software (SAS Institute, Cary, NC, USA).

RESULTS

IHC Staining of RAD18 in Clinical Gastric Cancer (GC) Specimens

RAD18 was mainly expressed in the nuclei of GC cells in the clinical samples. Higher RAD18 expression levels were detected in GC tissues than in normal gastric mucosa samples (Fig. 1a). Of the 96 GC specimens, 51 (53.1%) and 45 (46.9%) were categorized into the low (Fig. 1b, c) and high (Fig. 1d) RAD18 expression groups, respectively (Table 1).

Clinicopathological Significance of RAD18 Expression in Patients with GC

Table 1 depicts the relationships of RAD18 expression between various clinicopathological factors in this cohort of 96 patients and GC. A high level of RAD18 expression was significantly associated with the progression of lymph node metastasis ($p < 0.0001$), lymphatic invasion ($p = 0.0255$), venous invasion ($p < 0.0001$), and clinical stage ($p = 0.0253$) (Table 1).

Prognostic Significance of RAD18 Expression in Patients with GC

Our Kaplan–Meier analysis of data from 96 patients with GC revealed significantly lower overall and recurrence-free survival rates in the high RAD18 expression group than in the low RAD18 expression group ($p = 0.0318$, Fig. 2a; $p = 0.0103$, Fig. 2b). To confirm the prognostic significance of RAD18 expression in a larger cohort, we used the Kaplan–Meier plotter (www.kmplot.com), which includes the published microarray data of 631 GC samples. The findings from that analysis were consistent with the data from our cohort, and validated the association between a high level of RAD18 expression in GC samples and poor prognosis in a large database [hazard ratio 1.34, 95% confidence interval (CI) 1.07–1.68; $p = 0.0098$] (electronic supplementary figure 1).

Our univariate analysis of 96 GC patients identified a high level of RAD18 expression as a significant prognostic factor associated with poor survival [relative risk (RR) 2.01, 95% CI 1.06–3.96, $p = 0.0327$]. However, a multivariate analysis of the six factors identified as significant in the univariate analysis did not identify high RAD18 expression as an independent risk factor for poor overall survival (RR 1.08, 95% CI 0.5–2.23, $p = 0.8437$) (Table 2).

Functional Analysis of RAD18 in GC Cell Lines

Subsequently, we used Western blotting to determine that RAD18 protein was expressed in the MKN7, MKN45, MKN74, and GCIY GC cell lines (Fig. 3a), albeit at higher levels in MKN45 and GCIY cells. Accordingly, the two lines were selected for subsequent knockdown experiments to analyze the functional significance of RAD18 in cell proliferation, invasiveness, and cisplatin sensitivity. We used siRNA to silence RAD18 expression and used Western blotting to confirm that protein expression was suppressed (Fig. 3b). Furthermore, we observed significant reductions in cell proliferation and invasiveness in RAD18 siRNA-treated (suppressed) cells compared with control siRNA-treated cells (Fig. 3c, d).

RAD18 Regulated the Expression of DNA Damage Repair Proteins and was Associated with Cisplatin Sensitivity in GC Cell Lines

Western blotting was used to determine the protein expression of RAD18, phospho-H2A.X (DNA damage

TABLE 1 Clinicopathological significance of RAD18 expression of 96 GC patients

Variable	RAD18 expression		
	Low (n = 51)	High (n = 45)	p Value
Age, years (mean + SD)	65.5 ± 1.21	63.3 ± 1.29	0.212
Sex			
Male	40	39	0.288
Female	11	6	
Histology type			
Well, moderate	30	21	0.233
Poor, signet	21	24	
Tumor depth			
M, SM	14	7	0.155
MP, SS, SE, SI	37	38	
Lymph node metastasis			
Absent	31	10	0.0001*
Present	20	35	
Lymphatic invasion			
Absent	18	7	0.0255*
Present	33	38	
Venous invasion			
Absent	29	8	< 0.0001*
Present	22	37	
Stage			
I	29	12	0.0253*
II	8	10	
III	8	13	
IV	6	10	

GC gastric cancer, *SD* standard deviation, *M* mucosa, *SM* submucosa, *MP* muscularis propria, *SS* subserosa, *SE* serosa, *SI* invasion to the adjacent structures

*$p < 0.05$

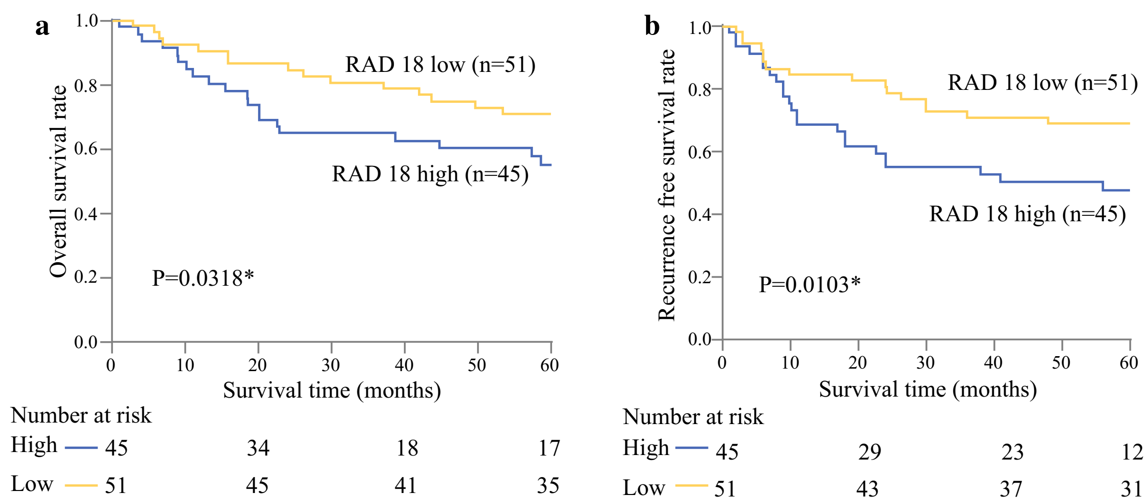

FIG. 2 Kaplan–Meier survival curves of patients with GC according to RAD18 expression. **a** Kaplan–Meier analysis of overall survival in our cohort of GC patients (n = 96). The analyses were based on RAD18 expression (p = 0.0318). **b** Kaplan–Meier analyses of recurrence-free survival (n = 96) according to RAD18 expression (p = 0.0103). The overall survival and recurrence-free survival rates were significantly lower in GC patients with high levels of RAD18 expression. *GC* gastric cancer

TABLE 2 Univariate and multivariate analysis of clinicopathological factors affecting overall survival rate following surgery in 96 patients with GC

Variable	Univariate analysis			Multivariate analysis		
	RR	95% CI	*p* Value	RR	95% CI	*p* Value
Age, years						
≤ 65 versus > 65	1.27	0.69–2.38	0.446	–	–	–
Sex						
Male versus female	1.06	0.49–2.08	0.864	–	–	–
Histology type						
Well, moderate versus poor, signet	2.34	1.26–4.50	0.0065*	1.45	0.69–3.09	0.3211
Tumor depth						
M, SM, MP versus SS, SE, SI	17.8	3.87–315.7	< 0.0001*	3.96	0.66–75.5	0.1384
Lymph node metastatic						
Absent versus present	3.73	1.89–9.03	< 0.0001*	2.34	1.07–5.75	0.0316*
Venous invasion						
Absent versus present	5.49	2.48–14.5	< 0.0001*	2.91	1.13–8.75	0.0253*
Peritoneal dissemination						
Absent versus present	7.55	2.97–16.9	0.0001*	9.41	3.10–25.9	0.0003*
RAD18 expression						
Low versus high	2.01	1.06–3.96	0.0327*	1.08	0.50–2.23	0.8437

RR relative risk, *CI* confidence interval, *M* mucosa, *SM* submucosa, *MP* muscularis propria, *SS* subserosa, *SE* serosa, *SI* invasion to the adjacent structures

*$p < 0.05$

marker), PCNA (essential component of DNA replication and repair), and mUB-PCNA (essential for DNA repair) in RAD18-suppressed GC cells after treatment with cisplatin and a DNA damage-inducing chemotherapeutic agent. Notably, cisplatin strongly induced the expression of phospho-H2A.X in the RAD18-suppressed cells relative to the control cells (Fig. 4a). In contrast, the levels of cisplatin-induced DNA damage repair proteins (PCNA and mUB-PCNA) were lower in RAD18-suppressed cells relative to control cells (Fig. 4). Finally, RAD18-suppressed cells exhibited significantly enhanced sensitivity to cisplatin compared with the control cells ($p < 0.01$) (Fig. 4a, b).

DISCUSSION

In this study, we demonstrated how high RAD18 expression is associated with cancer aggressiveness and poor prognosis in a cohort of 96 patients with GC. Moreover, we demonstrated reductions in the proliferative and invasive capacities of RAD18-suppressed GC cells in vitro. Furthermore, we observed increased chemosensitivity and DNA damage marker expression in RAD18-suppressed cells, which was mediated by the inhibition of cisplatin-induced DNA repair proteins such as mUB-PCNA.

DNA damage may be induced by environmental factors and endogenous cellular metabolites. Accordingly, cells have acquired DNA damage repair mechanisms that enable survival in the presence of lethal DNA damage. For example, mUB-PCNA coordinates with various DNA polymerases to regulate mutagenic translesion synthesis (TLS).[8] In humans, TLS requires the presence of selective mUB-PCNA sliding clamps that encircle the damaged DNA.[9] Accordingly, mUB-PCNA appears to be a critical initiator of postreplication repair.[10] In cancer research, mUB-PCNA plays an important role in chemotherapy-induced DNA damage repair. Moreover, RAD18 regulates the monoubiquitination of PCNA in both non-cancerous and cancer cells.[11–14] In this study, cisplatin treatment led to decreased mUB-PCNA expression and accumulated phosphorylated H2A.X and DNA damage marker in RAD18-suppressed GC cells relative to control cells. These findings highlight the importance of RAD18 as a component of DNA damage repair. In this regard, RAD18 might contribute to chemotherapy-induced DNA damage repair in GC cells by controlling mUB-PCNA, which in turn mediates TLS.

We further demonstrated that the suppression of RAD18 led to significant reductions in cellular viability and invasiveness, and increased chemosensitivity in GC cells. Consistent with our data, Zou and colleagues reported that RAD18 suppression led to decreased cell proliferation and

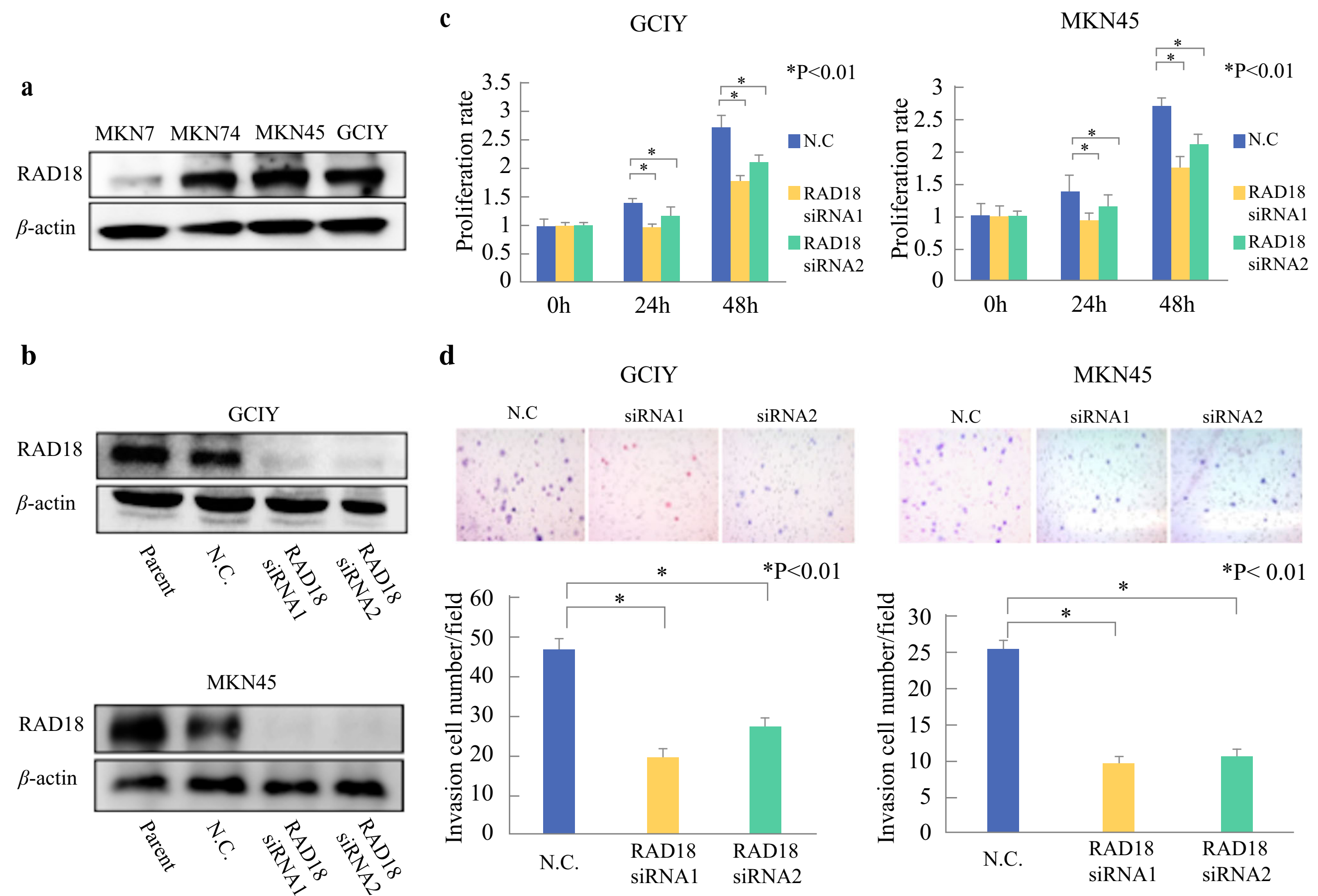

FIG. 3 Functional analysis of RAD18 in human GC cell lines. **a** RAD18 expression was evaluated in the GC cell lines MKN7, MKN74, MKN45, and GCIY by Western blotting. β-Actin was used as the loading control. **b** RAD18 suppression was evaluated in MKN45 and GCIY cells treated with RAD18 siRNAs by Western blotting. **c** The proliferation of MKN45 and GCIY cells after RAD18 siRNA treatment was evaluated using a Cell Counting Kit-8 kit. **d** RAD18 siRNA treatment significantly inhibited invasiveness, compared with the control. *GC* gastric cancer, *siRNA* small-interfering RNA, *N.C.* non-targeting control siRNA

increased chemosensitivity in esophageal squamous cell carcinoma cells.[6] These data strongly suggest that RAD18 is a promising therapeutic target in GCs characterized by high levels of proliferative ability, invasiveness, and chemoresistance.

The association of RAD18 with cancer aggressiveness has led to speculation that the associated gene may be important to the progression of several cancers, including GC. Therefore, the potential risk of adverse effects must be considered carefully in studies of molecular RAD18 inhibition therapy. One report described the significant induction of UV-induced skin carcinogenesis in RAD18 knockout mice, suggesting that the systemic suppression of this protein causes unexpected adverse effects, including the occurrence of a second cancer or the accumulation of DNA damage in non-cancerous tissues.[15] Cancer-specific RAD18 targeting would help to increase the chemosensitivity of refractory cancers while reducing the risk of severe adverse effects associated with systemic therapy.

Previous studies have determined how high RAD18 expression correlates with poor prognosis in other tumor types.[6,16–18] In this study, we first observed a statistical association of high RAD18 expression with poor survival in patients with GC. However, our multivariate analysis failed to identify a high RAD18 expression level as an independent prognostic factor. The close correlations identified between RAD18 and other strong prognostic factors for GC, including the progression of lymph node metastasis, lymphatic invasion, and venous invasion, may have prevented us from identifying RAD18 as an independent prognostic factor.

Our in vitro data showed a significant effect of RAD18 suppression strategy to improve the chemosensitivity in GC cells; therefore, we showed how RAD18 could be a predictive biomarker of chemosensitivity in clinical GC samples. We evaluated how prognosis relates to RAD18 expression in GC samples without neoadjuvant therapy of 34 patients who had recurrent diseases treated by a CDDP-

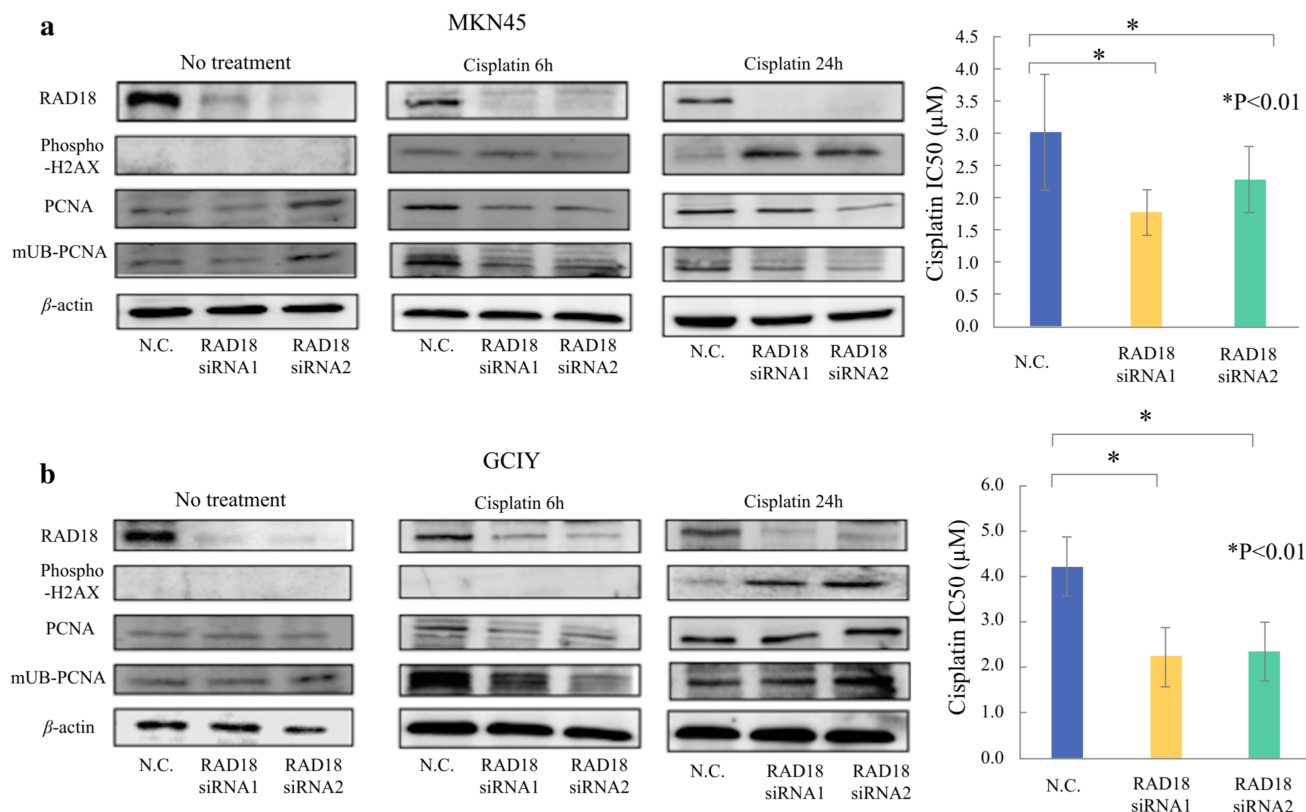

FIG. 4 Analysis of cisplatin chemosensitivity in RAD18-suppressed GC cells. **a** Expression of RAD18, phospho-H2AX, PCNA, and mUB-PCNA in MKN45, and **b** in GCIY cells treated with cisplatin for 6 or 24 h using Western blot. β-Actin was used as the loading control (left panel). The IC_{50} values for cisplatin were determined in GC cells after treatment with cisplatin for 72 h. The IC_{50} values were significantly lower in the RAD18 siRNA groups, compared with those in the NC siRNA groups (right panel). *GC* gastric cancer, *IC_{50}* half maximal inhibitory concentration, *siRNA* small-interfering RNA, *PCNA* proliferating cell nuclear antigen, *mUB-PCNA* monoubiquitinated PCNA, *N.C.* non-targeting control siRNA

based regimen after radical resection. Contrary to the expectations, we could not show the chemosensitivity predictive value of RAD18 evaluation in pretreatment GC samples (electronic supplementary figure 2). As mentioned above, RAD18 expression is caused by DNA damage inducers such as chemotherapy treatments, and the induced RAD18 can function in DNA damage repair. From these findings, it was suggested that the evaluation of RAD18 in clinical GC samples before chemotherapy might be insufficient for predicting the chemosensitivity. In this study, we wanted to emphasize the importance of RAD18 as a new target therapy candidate in GC, not as a chemosensitivity predictor.

This study had several limitations of note. First, the patient cohort was small and the data were collected retrospectively. Second, the included patients with GC were not consecutive because only resectable cases without neoadjuvant therapy were selected. Therefore, our data might not be generalizable to all patients with GC, including those with chemotherapy-treated unresectable tumors. In the future, large cohort, prospective studies of pretreatment biopsy tissues are warranted to establish the significance of a pretreatment RAD18 evaluation in cases of unresectable GC.

CONCLUSIONS

We observed correlations of a high RAD18 expression level with shorter overall and recurrence-free survival durations in patients with GC, suggesting that RAD18 is a marker of cancer aggressiveness and prognosis in this population. Our in vitro RAD18 siRNA analysis further clarified the role of RAD18 in the regulation of cisplatin sensitivity via the upregulation of cisplatin-induced DNA damage. Therefore, RAD18 may be a promising new molecular treatment target in chemoresistant refractory GCs.

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COMPLIANCE WITH ETHICAL STANDARDS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE This study conformed to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board for Clinical Research at Gunma University Hospital (Maebashi, Gunma, Japan; approval number HS2019-043). Patient consent was obtained using the opt-out method.

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