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High Expression of MRE11–RAD50–NBS1 Is Associated with Poor Prognosis and Chemoresistance in Gastric Cancer

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Abstract. *Background: The MRN complex of meiotic recombination 11 (MRE11), DNA repair protein Rad50 (RAD50) and Nijmegen breakage syndrome 1 (NBS1) proteins coordinate the detection and repair of DNA double-strand breaks (DSBs). DNA DSB repair-dependent chemoresistance likely has an effect on the treatment of human cancer. Materials and Methods: We investigated the expression of MRN complex in human gastric cancer (GC) tissues using immunohistochemistry and analyzed its clinical significance and prognostic relevance. Results: The expression of MRN complex was significantly associated with clinical factors including poorer prognosis and negatively associated with the expression of DNA damage marker phosphorylated H2A histone family, member X (γH2AX) in the nucleus. In the biopsy specimens, low expression of MRE11 correlated with good response to chemotherapy and surgical resection after down-staging by chemotherapy. Furthermore, the expression levels of MRE11 and RAD50 were independent predictors of surgical resection after chemotherapy. Conclusion: The high expression of MRN complex constituents could be a predictor for poor prognosis and chemoresistance in GC.*

Gastric cancer (GC) is the fifth most common cancer worldwide, with nearly one million diagnoses annually (1). Treatment with aggressive and adjuvant chemotherapy in advanced GC has led to improved survival rates, but the prognosis for patients with refractory GC with unresectable regions remains poor and unsatisfactory (2). Therefore, further research is required to identify new therapeutic targets capable of overcoming chemoresistance in order to improve the prognosis in these patients.

DNA damage is known to be associated with chemoresistance, cancer progression, genomic instability, and carcinogenesis (3-5). DNA double-strand breaks (DSBs) are one of the most severe threats to cancer cell survival, and are repaired by the mechanisms of homologous recombination and non-homologous end-joining. Moreover, DNA DSB repair genes are often activated in several refractory types of cancer, including GC, and are reported to convey chemo- and radiotherapy resistance (6, 7). As such, targeting DSB repair genes presents as a promising strategy for eliminating chemoresistant cancer cells.

DSB repair is initiated through the combined efforts of ataxia telangiectasia mutated (ATM) and a protein complex consisting of meiotic recombination 11 (MRE11), RAD50 double strand break repair protein (RAD50) and Nijmegen breakage syndrome 1 (NBS1) proteins to form the MRE11–RAD50–NBS1 (MRN) complex (8, 9). In the nucleus, the MRN complex binds to sites of DNA DSBs where it recruits

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and activates ATM, which can then phosphorylate multiple substrates including phosphorylated H2A histone family, member X (γH2AX); p53 binding protein (153BP1); structural maintenance of chromosomes protein 1 (SMC1); breast cancer, early-onset 1 (BRCA1); and checkpoint kinase 2 (CHK2) to trigger cell-cycle arrest and apoptosis of cancer cells (10-15). Notably, the MRN complex is associated with increased chemoresistance in several cancer types *via* its role in DNA DSB repair (16-19). Matsutani *et al*. reported that the expression levels of MRE11, RAD50, and NBS1 were higher in GC tissues when compared to corresponding non-cancerous tissues (20). However, the role of the MRN complex in cancer progression, prognosis, and chemosensitivity has not yet been investigated in clinical GC samples.

The purpose of this study was to clarify the clinical significance of MRN complex in biopsy specimens from 210 patients undergoing radical resection and 78 with unresectable tumors obtained at initial diagnosis from chemotherapy-naïve patients with GC. Specimens were subjected to immunohistochemistry to assess MRE11, RAD50 and NBS1 expression in order to evaluate their effect on clinicopathological factors, prognosis, and clinical response after chemotherapy.

Materials and Methods

Patients and clinical samples. Four serial surgical specimens for tissue microarrayevaluation of MRE11, RAD50, NBS1 and γH2AX expression were obtained from each of 210 patients with GC who underwent radical resection. Patient characteristics are described in Table I.

Three serial biopsy specimens were obtained from each of 78 patients with GC (10 stage III, 68 stage IV) who presented with unresectable GC at initial diagnosis. Twenty-one out of 78 cases were treated with surgery after down-staging by chemotherapy. The remaining 57 cases remained inoperable after down-staging by chemotherapy. Forty-seven out of the 78 patients received S-1 (tegafur-gimeracil-oteracil potassium) orally on days 1-14 plus paclitaxel as an intravenous infusion on days 1, 8, and 15 of a 4-week cycle. The remaining 31 patients received S-1 orally on days 1-21 plus cisplatin as an intravenous infusion on day 8 of a 5-week cycle(21). Other characteristics are described in Table II.

All clinical GC samples were obtained at Gunma University Hospital, Department of General Surgical Science, from January 1999 and March 2006, and were used in accordance with institutional guidelines and the Helsinki Declaration after obtaining written informed consent from all participants. The clinicopathological factors were obtained from pathological reports and medical records including age, gender, tumor location, histology, Lauren's classification, tumor depth, lymph node metastasis, lymphatic invasion, venous invasion, clinical stage, first-line chemotherapy regimen, and clinical response by first-line chemotherapy. 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 (22).

Tissue microarrays. Clinical samples were formalin-fixed and paraffin-embedded, and then stored in the archives of the Clinical Department of Pathology, Gunma University Hospital. For each patient, one paraffin block containing representative non-necrotic tumor areas was selected. Two GC tissue cores (2.0-mm diameter per tumor) were punched out from the representative areas near the invasive front and transferred into the paired recipient paraffin block using a tissue array instrument (Beecher Instruments, Silver Spring, MD, USA).

Immunohistochemistry. Immunohistochemical staining was performed with 2-μm-thick sections. All sections were incubated at 60˚C for 60 min and deparaffinized in xylene, rehydrated, and then incubated with fresh 0.3% hydrogen peroxide in 100% methanol for 30 min at room temperature to block endogenous peroxidase activity. After rehydration through a graded series of ethanol solutions, antigen retrieval was carried out in Immunosaver (NJ15T, NEM, Tokyo, Japan) at 98-100˚C for 30 min, and then sections were passively cooled to room temperature. After rinsing in 0.1 M phosphate-buffered saline (PBS, pH 7.4), sections were incubated in Protein Block Serum-Free Reagent (DAKO, Carpinteria, CA, USA) for 30 min to block non-specific binding sites. The sections were then incubated with rabbit monoclonal antibody to MRE11 (ab109623; Abcam, Cambridge, MA, USA), mouse monoclonal antibody to RAD50 (ab87918; Abcam), rabbit monoclonal antibody NBS1 (ab175800; Abcam) and mouse monoclonal antibody to γH2AX (phospho-S139) (ab26350; Abcam) at a dilution of 1:300 in PBS containing 0.1% bovine serum albumin overnight at 4˚C and then incubated at room temperature for 30 min. The reaction 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. Negative controls were incubated without primary antibody, and no detectable staining was evident.

Evaluation of immunostaining. Immunohistochemical slides were scanned and evaluated by two experienced researchers (Cohen's κ=0.839). The intensity of staining for the nuclear MRN complex and γH2AX was scored as follows: 0: no staining; 1+: weak staining; 2+: moderate staining; 3+: strong staining. The percentage of nuclear-stained cells was calculated by examining at least 10^3 cancer cells in five representative areas. The percentage of staining of nuclear MRN complex and γH2AX was scored as follows: 0: no staining; 1+: 1-25%; 2+: 26-50%; 3+: 51-100%. The final score was defined as the percentage score multiplied by the intensity score (0; 1+; 2+; 3+; 4+; 6+; 9+). Nuclear immunoreactivity of the MRN complex and γH2AX was scored on a scale of 0–9+ with scores of 0–4+, and 5–9+ defined as low and high nuclear expression, respectively(23).

Fluorescent immunohistochemistry. The sections were prepared, and endogenous peroxidase was blocked as described above. The sections were then boiled in citrate buffer (pH 6.4) for 15 min in a microwave, and then incubated with fresh 0.3% hydrogen peroxide in 100% methanol for 30 min at room temperature to block endogenous peroxidase activity. Nonspecific binding sites were blocked by incubation with Protein Block Serum-Free Reagent for

Table I. *Association of meiotic recombination 11 (MRE11), DNA repair protein Rad50 (RAD50) and Nijmegen breakage syndrome 1 (NBS1) complex expression in 210 gastric cancer surgical samples with clinicopathological factors.*

Factor	MRE11			RAD50			NBS1		
	Low (n=90)	High (n=120)	*p*-Value	Low (n=54)	High (n=156)	*p*-Value	Low (n=94)	High (n=116)	*p*-Value
Age (mean±SD), years	64±12.15	65±10.32	0.5694	65±10.89	63±11.38	0.3056	63±10.66	65±11.09	0.467
Gender, n (%)									
Male	65 (72.2)	82 (68.3)	0.5428	38 (70.4)	109 (69.9)	0.9451	72 (76.6)	75 (64.7)	0.0604
Female	25 (27.8)	38 (31.7)		16 (29.6)	47 (30.1)		22 (23.4)	41 (35.3)	
Histology, n (%)									
Well, moderate	35 (38.9)	44 (36.7)	0.7422	18 (33.3)	61 (39.1)	0.4507	41 (43.6)	38 (32.8)	0.1063
Poor, signet	55 (61.1)	76 (63.3)		36 (66.7)	95 (60.9)		53 (56.4)	78 (67.2)	
Lauren's classification, n (%)									
Diffuse	48 (53.3)	65 (54.2)	0.5201	32 (59.3)	81 (51.9)	0.4789	42 (44.7)	71 (61.2)	0.0108*
Intestinal	34 (37.8)	39 (32.5)		18 (33.3)	55 (35.3)		43 (45.7)	30 (25.9)	
Mixed	8 (8.9)	16 (13.3)		4 (7.4)	20 (12.8)		9 (9.6)	15 (12.9)	
Tumor depth, n (%)									
SM, MP	30 (33.3)	25 (20.8)	0.0415*	17 (31.5)	38 (24.4)	0.3049	26 (27.7)	29 (25.0)	0.6629
SS, SE, SI	60 (66.7)	95(79.2)		37 (68.5)	118 (75.6)		68 (72.3)	87 (75.0)	
Lymph node metastasis, n (%)									
Absent	38 (42.2)	29 (24.2)	0.0055*	24 (44.4)	43 (27.6)	0.0218*	35 (37.2)	32 (27.6)	0.1358
Present	52 (57.8)	91 (75.8)		30 (55.6)	113 (72.4)		59 (62.8)	84 (72.4)	
Lymphatic invasion, n (%)									
Absent	12 (13.3)	7 (5.8)	0.0608	7 (13.0)	12 (7.7)	0.2445	11 (11.7)	8 (6.9)	0.2274
Present	78 (86.7)	113 (94.2)		47 (87.0)	144 (92.3)		83 (88.3)	108 (93.1)	
Venous invasion, n (%)									
Absent	71 (78.9)	81 (67.5)	0.0677	42 (77.8)	110 (70.5)	0.3034	72 (76.6)	80 (69.0)	0.2188
Present	19 (21.1)	39 (31.5)		12 (22.2)	46 (29.5)		22 (23.4)	36 (32.0)	
Clinical stage, n (%)									
I	17 (18.9)	16 (13.3)	0.0074*	11 (20.4)	22 (14.1)	0.0751	17 (18.1)	16 (13.8)	0.1653
II	39 (43.3)	30 (25.0)		23 (42.6)	46 (29.5)		35 (37.2)	34 (29.3)	
III	26 (28.9)	56 (46.7)		17 (31.5)	65 (41.7)		35 (37.2)	47 (40.5)	
IV	8 (8.9)	18 (15.0)		3 (5.5)	23 (14.7)		7 (7.5)	19 (16.4)	
γH2AX, n (%)									
Low	56 (62.2)	92 (76.7)	0.0232*	19 (35.2)	129 (82.7)	<0.0001*	51 (54.3)	97 (83.6)	<0.0001*
High	34 (37.8)	28 (23.3)		35 (64.8)	27 (17.3)		43 (45.7)	19 (16.4)	
RAD50, n (%)									
Low	36 (40.0)	18 (15.0)	<0.0001*						
High	54 (60.0)	102 (85.0)							
NBS1, n (%)									
Low	54 (60.0)	40 (33.3)	<0.0001*						
High	36 (40.0)	80 (66.7)							

SM: Submucosal invasion; MP: *muscularis propria* invasion; SS: subserosal invasion; SE: serosal invasion; SI: surrounding organ invasion, γH2AX: DNA damage marker phosphorylated H2A histone family, member X. *Significant difference.

30 min, and the sections were incubated with the above-mentioned primary antibodies against NBS1, RAD50, and MRE11 diluted in PBS containing 0.01% bovine serum albumin for 3 h at room temperature. Multiplex covalent labeling with tyramide signal amplification (Opal™ 3-Plex Kit; PerkinElmer, Waltham, MA, USA) was performed according to the manufacturer's protocol. All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and examined under an All-in-One BZ-X710 fluorescence microscope (KEYENCE Corporation).

Statistical analysis. Statistically significant differences were analyzed with the Student's *t*-test and χ^2 test for continuous and categorical variables, respectively. Survival curves were generated by Kaplan–Meier analysis and differences were examined by log-rank testing. In addition, univariate and multivariate survival analyses were performed using Cox's proportional hazards model for survival and logistic regression model for radical surgery after down-staging. Values of $p<0.05$ and $p<0.1$ were considered as statistically significant and indicative of trending data, respectively. All statistical analyses were performed using JMP 9.0 software (SAS Institute Inc., Cary, NC, USA).

Results

Nuclear expression of MRN complex and γH2AX in clinical GC samples. We used immunohistochemistry to investigate

Table II. *Univariate and multivariate analyses of clinicopathological factors according to the Japanese Classification of Gastric Carcinoma affecting overall survival in 210 patients with gastric cancer and disease-free survival in 186 patients with primary gastric cancer. Protein expression was determined from surgical specimens.*

Clinicopathological variable	Univariate			Multivariate (MRE11)			Multivariate (RAD50)			Multivariate (NBS1)		
	RR	95% CI	*p*-Value	RR	95% CI	*p*-Value	RR	95% CI	*p*-Value	RR	95% CI	*p*-Value
Overall survival												
Age: <65/≥65 years	1.17	0.78-1.77	0.4467	-	-	-	-	-	-	-	-	-
Gender: Male/female	1.17	0.76-1.86	0.4908	-	-	-	-	-	-	-	-	-
Histology grade:												
Differentiated/undifferentiated	0.73	0.47-1.12	0.1546	-	-	-	-	-	-	-	-	-
Tumor depth: SM, MP/SS, SE, SI	3.63	2.38-5.67	<0.0001*	1.24	0.62-2.65	0.5507	1.26	0.62-2.69	0.5348	1.00	0.69-1.46	1.0003
Lymph node metastasis: Absent/present	2.65	1.60-4.63	<0.0001*	0.73	0.34-1.62	0.4372	0.72	0.33-1.58	0.4074	1.12	0.73-1.73	0.5868
Lymphatic invasion: Absent/present	3.75	1.41-15.27	0.0051*	1.86	0.61-8.09	0.2996	1.85	0.60-8.03	0.3063	1.00	0.59-1.76	0.9833
Venous invasion: Absent/present	2.20	1.43-3.34	0.0004*	1.72	1.10-2.64	0.0177*	1.74	1.12-2.65	0.0141*	1.29	0.93-1.76	0.1195
Stage: I, II/III, IV	4.11	2.60-6.74	<0.0001*	3.83	1.88-8.44	0.0001*	3.68	1.83-7.97	0.0001*	1.50	0.99-2.31	0.0537
MRE11expression: Low/high	1.56	1.03-2.41	0.0367*	0.99	0.64-1.57	0.9661	-	-	-	-	-	-
RAD50 expression: Low/high	1.81	1.10-3.17	0.0185*	-	-	-	1.54	0.93-2.71	0.0935	-	-	-
NBS1 expression: Low/high	1.74	1.15-2.70	0.0088*	-	-	-	-	-	-	1.70	1.28-2.27	0.0003*
Disease-free survival (stage I, II, III)												
Age: <65/≥65 years	1.22	0.80-1.89	0.3484	-	-	-	-	-	-	-	-	-
Gender: Male/female	1.18	0.49-0.74	0.4968	-	-	-	-	-	-	-	-	-
Histology grade:												
Differentiated/undifferentiated	1.28	0.2-0.82	0.27	-	-	-	-	-	-	-	-	-
Tumor depth: SM, MP/SS, SE, SI	0.33	0.18-0.57	<0.0001*	-	-	-	-	-	-	2.05	1.30-3.28	0.561
Lymph node metastasis: Absent/present	2.75	1.66-4.85	<0.0001*	-	-	-	-	-	-	1.03	0.51-2.11	0.5021
Lymphatic invasion: Absent/present	5.96	1.88-36.21	0.0007*	-	-	-	-	-	-	2.18	0.73-9.36	0.205
Venous invasion: Absent/present	2.02	1.26-3.14	0.0037*	-	-	-	-	-	-	1.78	1.19-2.63	0.344
Stage: I, II/III, IV	3.01	1.95-4.74	<0.0001*	-	-	-	-	-	-	2.04	1.06-4.08	0.0953
MRE11expression: Low/high	1.39	0.81-2.58	0.2397	-	-	-	-	-	-	-	-	-
RAD50 expression: Low/high	1.25	0.81-1.94	0.3264	-	-	-	-	-	-	-	-	-
NBS1 expression: Low/high	3.00	1.29-3.55	0.0023*	-	-	-	-	-	-	1.45	0.99-2.14	0.0008*

MRE11: Meiotic recombination 11; RAD50: DNA-repair protein Rad50; NBS1: Nijmegen breakage syndrome 1; RR: relative risk; CI: confidence interval; SM: submucosal invasion; MP: *muscularis propria* invasion; SS: subserosal invasion; SE: serosal invasion; SI: surrounding organ invasion. *Significant result.

the nuclear expression of MRE11, RAD50, NBS1, and γH2AX in four serial surgical specimens obtained from 210 GC samples. Results were as follows: high *vs.* low expression: MRE11: 120 *vs.* 90 (57.1% *vs.* 42.9%); RAD50: 156 *vs.* 54 low (74.3% *vs.* 25.7%); NBS1: 116 *vs.* 94 low (55.2% *vs.* 44.8%); γH2AX: 62 *vs.* 148 low (29.5% *vs.* 70.5%). Representative results of the immunohistochemistry are shown in Figure 1.

Our analysis revealed enhanced nuclear expression of DNA damage marker γH2AX in specimens with low expression of MRN complex. Conversely, nuclear γH2AX expression was decreased in specimens with high expression of the MRN complex (Figure 1A).

Association between MRN complex expression and clinicopathological factors of 210 patients with GC. High nuclear MRE11 expression was significantly positively associated with tumor depth (p=0.0415), lymph node metastasis (p=0.0055), clinical stage (p=0.0074), low nuclear expression of γH2AX (p=0.0232), and nuclear accumulation of RAD50 (p<0.0001) and NBS1 (p<0.0001). Co-expression of MRN complex was validated in identical GC tissue using multi-fluorescent immunohistochemistry (Figure 2). High nuclear RAD50 expression was significantly positively associated with lymph node metastasis (p=0.0218) and low nuclear expression of γH2AX (p<0.0001). High nuclear NBS1 expression was significantly associated with diffuse-type GC (p=0.0108) and low nuclear expression of γH2AX (p<0.0001) (Table I).

Prognostic significance of nuclear MRN complex and γH2AX expression in GC. The overall survival rates of patients with GC assigned to the high MRN/low γH2AX group were significantly lower than that of those assigned to the low MRN/high γH2AX group (survival according to: MRE11, p=0.0385; RAD50, p=0.0242; NBS1, p=0.0094;

and γH2AX, p=0.0003) (Figure 1B). This finding also extended to patients with GC without synchronous unresectable metastases (stage I-III, n=184), where the disease-free survival rates for patients assigned to the high MRN/low γH2AX were lower than those of patients assigned to the low MRN/high γH2AX group (survival according to: MRE11, p=0.0729; RAD50, p=0.0402; NBS1, p=0.0032; and γH2AX, p=0.0204) (Figure 1B). Multivariate analysis revealed that high nuclear expression of NBS1 was an independent prognostic factor (relative risk (RR)=1.70, 95% confidence interval (CI)=1.28–2.27, p=0.0003) (Table II). Moreover, high nuclear expression of NBS1 was also an independent predictor of recurrence after surgical resection (RR=1.45, CI=0.99-2.14, p=0.0008) (Table II).

Association of MRN complex expression and clinicopathological features of unresectable GC. We first confirmed that the evaluation of MRN complex expression by immunohistochemistry was also possible using the biopsy samples (Figure 3). Our analyses revealed that high nuclear MRE11 expression (n=56) in 78 biopsy samples was significantly associated with poor clinical response to first-line chemotherapy (p=0.0436) and high nuclear RAD50 expression (n=59) may be associated with histological type (p=0.0856), tumor depth (p=0.0768) and clinical response (p=0.0803) (Table III). Moreover, improved surgical resection after down-staging using first-line chemotherapy (21/78, 26.9%) was associated with the low nuclear expression of MRE11, RAD50 and NBS1 (p<0.0001, p<0.0001, p=0.0501 respectively) (Table III).

Relationship of MRN complex expression and chemotherapeutic response in unresectable GC. The overall survival rates of patients with GC assigned to the high MRN group were significantly lower than those assigned to the low MRN group (survival according to: MRE11, p<0.0001; RAD50, p=0.0005; NBS1, p=0.0186) (Figure 4A), as were those of patients with GC treated with cisplatin (n=31) (survival according to: MRE11, p=0.0073; RAD50, p=0.0658; NBS1, p=0.0314) (Figure 4B) and those patients treated by paclitaxel (n=47) (survival according to: MRE11, p=0.0042; RAD50, p=0.0027; NBS1, p=0.0962) (Figure 4C).

Multivariate analysis revealed that high expression of nuclear MRN complex in unresectable GC biopsy samples was an independent prognostic factor (MRE11: RR=4.18, 95% CI=2.03-9.80, p<0.0001; RAD50: RR=3.73, 95% CI=1.22-8.17, p<0.0001; NBS1, RR=2.78, 95% CI=1.21-8.04, p=0.0135) (Table IV). Moreover, multivariate analysis for conversion to surgery after chemotherapy revealed that low expression of MRE11 and RAD50 was an independent predictor for surgical resection after down-staging (MRE11: OR=21.17, CI=5.83-94.83, p<0.0001; RAD50: OR=17.98, CI=4.78-84.25, p<0.0001) (Table V).

Discussion

In our current study, we found that the nuclear accumulation of MRN complex in GC tissues was associated with cancer progression, low nuclear expression of the DNA damage marker γH2AX, and poor prognosis. In particular, high NBS1 expression in GC was an independent prognostic factor. Moreover, high MRN complex expression in biopsy samples from patients with unresectable GC was associated with poor prognosis, chemotherapeutic response, and surgical resection after down-staging by chemotherapy.

The clinical significance of MRE11, RAD50, and NBS1 expression as clinical markers has already been reported. Among them, high MRE11 is associated with poor prognosis and chemoresistance in colon cancer and breast cancer(24, 25). Moreover, high tumor NBS1 expression is a poor prognostic factor in breast cancer, prostate cancer, acute myeloid leukemia, and oral squamous cell carcinoma (25-28). On the other hand, MRN complex expression is also associated with lower local recurrence rate after surgery in breast and esophageal cancer (29, 30). Moreover, Teo *et al*. reported that the sensitivity to radical radiotherapy is associated with MRE11 variant status in bladder cancer(31). Radiation therapy is rarely used as adjuvant therapy in current GC practice; therefore, we feel that our data for the association between MRN complex expression and poor prognostic factors is consistent with previous studies without adjuvant radiation treatments. This report is the first validation of relationships between MRN complex expression and chemosensitivity not only in surgical GC specimens, but also in biopsy samples from chemotherapy-naïve patients presenting with unresectable GC at initial diagnosis.

In this study, MRN complex expression was associated with several clinicopathological factors in patients with GC. From previous reports, MRN complex functions in DNA DSBs repair, as well as in the regulation of proliferation and telomere stability (11, 25, 32, 33). Moreover, NBS1 overexpression in head and neck cancer was reported to function as an inducer of epithelial–mesenchymal transition, which enhances the ability of cells to undergo migration and invasion, and acquire cancer stem cell-like properties (34). Therefore, it was suggested that MRN complex expression would be associated with cancer progression and poor prognosis due to its ability to promote DNA DSB repair and increase the malignant potential of GC cells.

DNA repair machinery plays an important role in chemorefractive cancer; therefore, many researchers have focused on these proteins as therapeutic targets. Among them, we examined the MRN complex. MRN complex inhibition is reported to induce antitumor effect in several cancer types (16, 35, 36). Moreover, MRN complex expression is known to be associated with resistance to hyperthermia and radiation

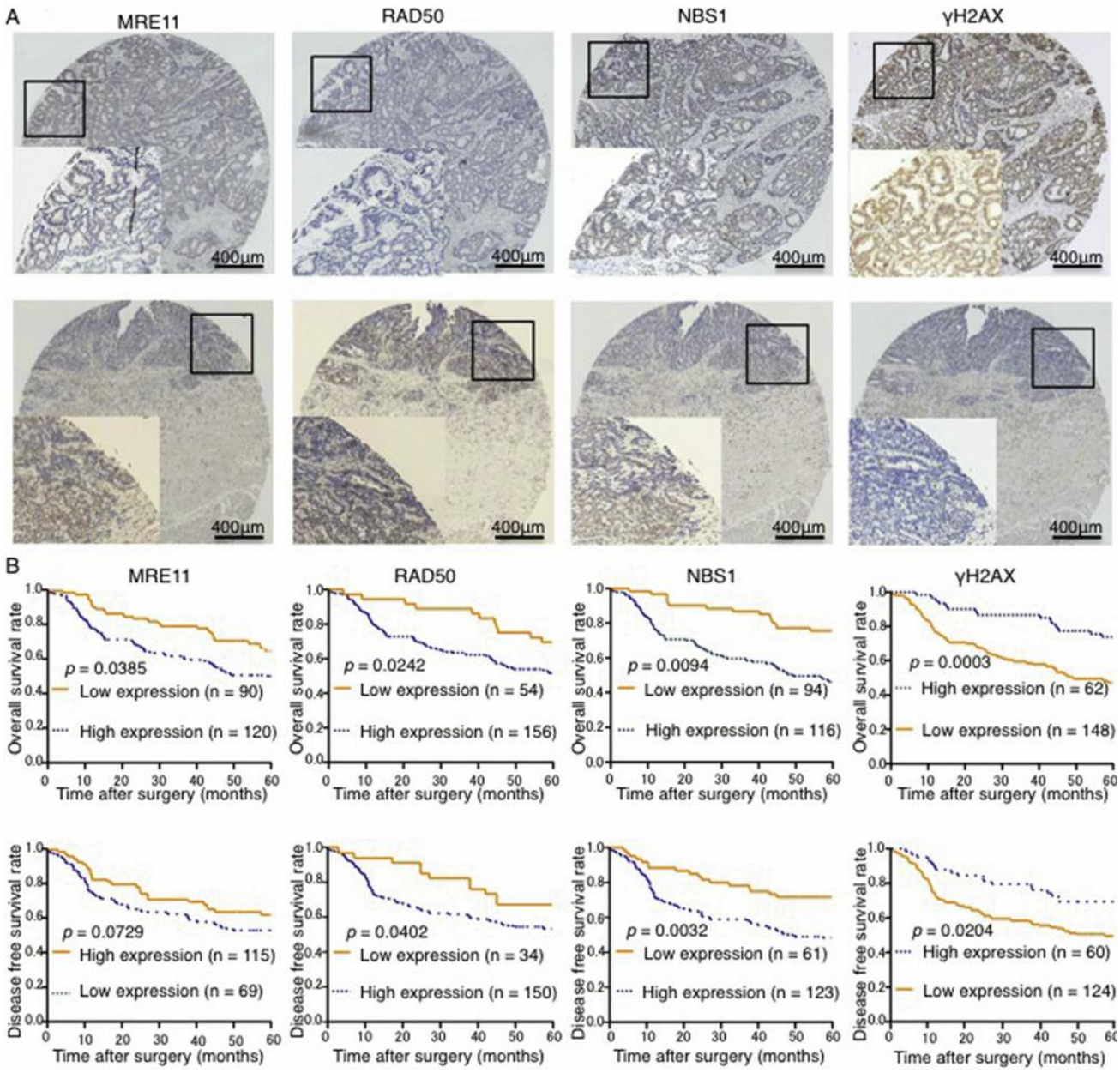

Figure 1. *Immunohistochemical staining of meiotic recombination 11, DNA-repair protein Rad50 and Nijmegen breakage syndrome 1 (MRE11–RAD50–NBS1 (MRN) complex and phosphorylated H2A histone family, member X (γH2AX) in primary gastric cancer (GC). A: Representative images of GC with a low nuclear level of MRE11, RAD50 and NBS1, and nuclear accumulation of DNA damage marker γH2AX (upper panel). and GC with a high nuclear level of MRE11, RAD50 and NBS1, and decreased expression of γH2AX (lower panel). B: Overall survival curve of 210 patients with GC (upper panel) and disease-free survival curve of 184 patients with GC (lower panel) according to expression of MRE11, RAD50, NBS1 and γH2AX.*

therapy by reducing DNA damage by these therapeutic modalities (37, 38). In this study, we found that GC samples with high MRN complex expression exhibited a low nuclear expression of the DNA damage marker γH2AX. Thus, the MRN complex might function to reduce DNA damage in patients with GC. Altogether, targeting the MRN complex is expected to be a promising therapeutic tool to overcome GC resistance to multimodal therapies.

In this study, we used biopsy samples to evaluate the expression of MRN complex and the chemotherapeutic

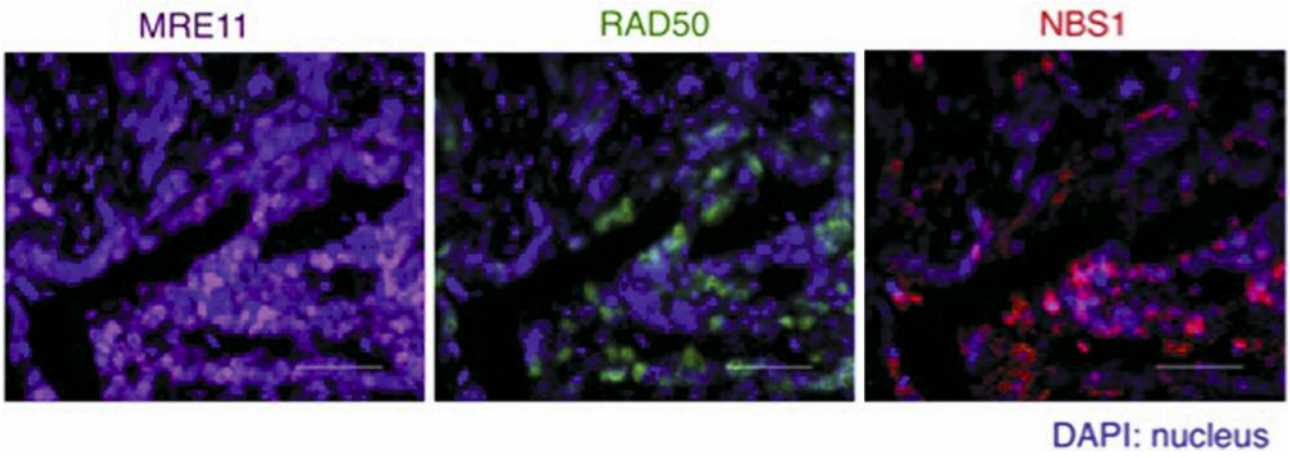

Figure 2. *Fluorescence immunohistochemical analysis of meiotic recombination 11 (MRE11), DNA repair protein Rad50 (RAD50) and Nijmegen breakage syndrome 1 (NBS1) expression in gastric cancer (GC) tissues. Representative GC tissue was immunostained with the antibodies against MRE11 (purple), RAD50 (green), and NBS1 (red). All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Scale bar= 50 μm.*

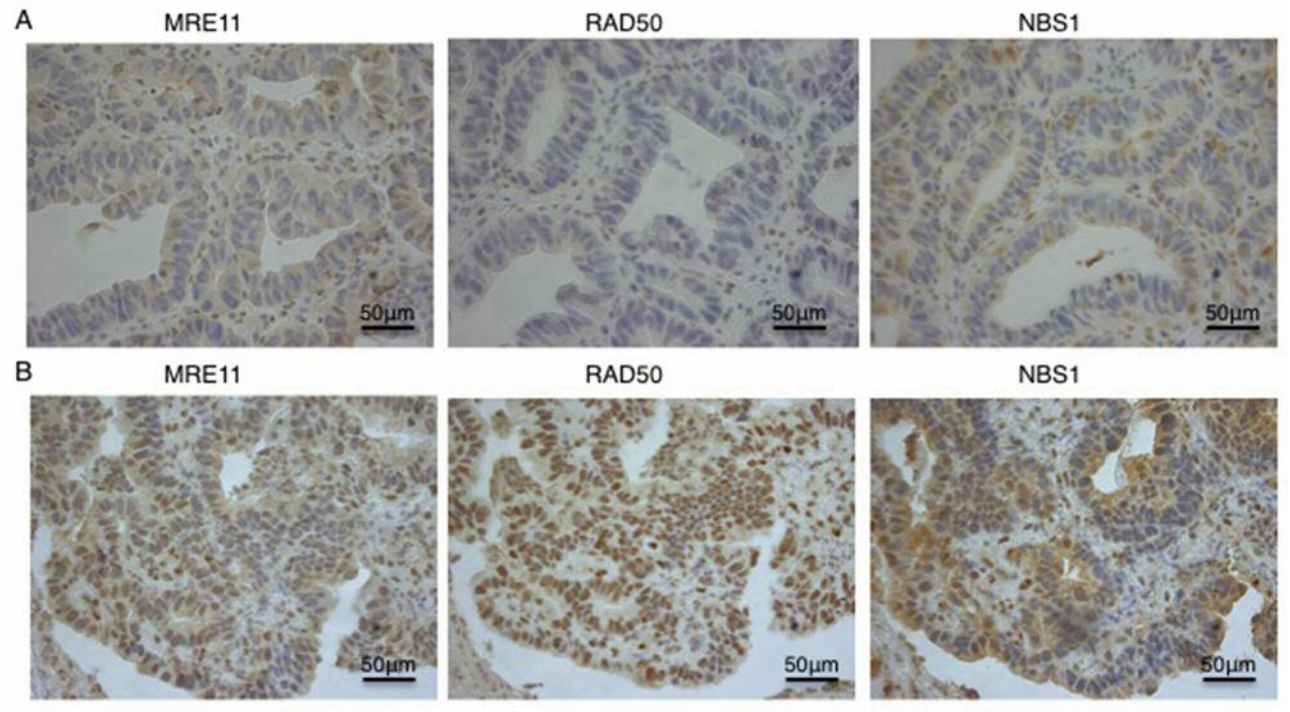

Figure 3. *A: Representative images of biopsy samples from a patient with unresectable gastric cancer (GC) with low-level nuclear expression of meiotic recombination 11 (MRE11) in tumor demonstrating decreased nuclear expression of DNA repair protein Rad50 (RAD50) and Nijmegen breakage syndrome 1 (NBS1). B: Representative images of the biopsy sample from a patient with unresectable GC with high nuclear MRE11 expression in tumor and enhanced nuclear expression of RAD50 and NBS1.*

response in patients with unresectable GC. Thus, this may bias the results of our study. However, our biopsy samples were adequate for evaluating the immunohistochemical staining of the MRN complex because of the non-heterogeneous expression of MRN complex in our surgical samples.

In conclusion, the nuclear accumulation of the MRN complex in surgical and biopsy samples from patients with GC was associated with cancer progression, reduced DNA damage, poor prognosis, and chemoresistance. These data suggest that the MRN complex might be a promising marker

Table III. *Association of meiotic recombination 11 (MRE11), DNA repair protein Rad50 (RAD50) and Nijmegen breakage syndrome 1 (NBS1) complex expression in 78 gastric cancer biopsy samples with clinicopathological factors.*

Clinicopathologic variable	MRE11 expression			RAD50 expression			NBS1 expression		
	Low (n=22)	High (n=56)	*p*-Value	Low (n=19)	High (n=59)	*p*-Value	Low (n=12)	High (n=66)	*p*-Value
Age (mean±SD), years	60.3±9.1	63.7±10.4	0.1881	61.2±9.7	63.2±10.2	0.4418	55.8±9.3	64±9.7	0.0078*
Gender, n (%)									
Male	13 (59.1)	40 (71.4)	0.2934	10 (52.6)	43 (72.9)	0.1	6 (50.0)	47 (71.2)	0.1475
Female	9 (40.9)	16 (28.6)		9 (47.4)	16 (27.1)		6 (50.0)	19 (28.8)	
Histology, n (%)									
Well, moderate	10 (45.5)	22 (39.3)	0.6182	11 (57.9)	21 (35.6)	0.0856	4 (33.3)	28 (42.4)	0.5559
Poor, signet	12 (54.5)	34 (60.7)		8 (42.1)	38 (64.4)		8 (66.7)	38 (57.6)	
Tumor depth, n (%)									
SS	5 (22.7)	7 (12.5)	0.5291	6 (31.6)	6 (10.2)	0.0768	2 (16.7)	10 (15.2)	0.5739
SE	14 (63.7)	40 (71.4)		11 (57.9)	43 (72.9)		7 (58.3)	47 (71.2)	
SI	3 (13.6)	9 (16.1)		2 (10.5)	10 (16.9)		3 (25.0)	9 (13.6)	
Lymph node metastasis, n (%)									
Absent	1 (4.6)	5 (8.9)	0.5133	2 (10.5)	4 (6.8)	0.594	1 (8.3)	5 (7.6)	0.9278
Present	21 (95.4)	51 (91.1)		17 (89.5)	55 (93.2)		11 (91.7)	61 (92.4)	
Hepatic metastases, n (%)									
Absent	20 (90.9)	47 (83.9)	0.4254	17 (89.5)	50 (84.8)	0.6066	11 (91.7)	56 (84.9)	0.5325
Present	2 (9.1)	9 (16.1)		2 (10.5)	9 (15.2)		1 (8.3)	10 (15.1)	
Distant metastases, n (%)									
Absent	17 (77.3)	45 (80.4)	0.7614	15 (79.0)	47 (79.7)	0.9466	11 (91.7)	51 (77.3)	0.256
Present	5 (22.7)	11 (19.6)		4 (21.1)	12 (20.3)		1 (8.3)	15 (22.7)	
Peritoneal metastases, n (%)									
Absent	14 (63.6)	32 (57.1)	0.5998	12 (63.2)	34 (57.6)	0.6699	8 (66.7)	38 (57.6)	0.5559
Present	8 (36.4)	24 (42.9)		7 (36.8)	25 (42.4)		4 (33.3)	28 (42.4)	
Clinical response, n (%)									
Partial response	18 (81.8)	28 (50.9)	0.0436*	15 (79.0)	31 (53.5)	0.0803	9 (75.0)	37 (56.9)	0.4089
Stable disease	2 (9.1)	15 (27.3)		3 (15.8)	14 (24.1)		1 (8.3)	16 (24.6)	
Progressive disease	2 (9.1)	12 (21.8)		1 (5.2)	13 (22.4)		2 (16.7)	12 (18.5)	
Chemotherapy, n (%)									
Cisplatin	10 (45.5)	21 (37.5)	0.5183	10 (52.6)	21 (35.6)	0.1869	6 (50.0)	25 (37.9)	0.43
Paclitaxel	12 (54.5)	35 (62.5)		9 (47.4)	38 (64.4)		6 (50.0)	41 (62.1)	
Surgical status after CTx									
No surgery	7 (31.8)	50 (89.3)	< 0.0001*	6 (31.6)	51 (86.4)	< 0.0001*	6 (50.0)	51 (77.3)	0.0501
Surgery	15 (68.2)	6 (10.7)		13 (68.4)	8 (13.6)		6 (50.0)	15 (22.7)	

SM: Submucosal invasion; MP: *muscularis propria* invasion; SS: subserosal invasion; SE: serosal invasion; SI: surrounding organ invasion, CTx: chemotherapy. *Significant difference.

for poor prognosis and chemoresistance in GC. Moreover, MRN complex inhibition may be an effective therapeutic approach to overcome chemoresistance in GC by blocking the DNA DSB repair; however, further studies are required to evaluate the full role of the MRN complex and its clinical application for GC treatment.

Conflicts of Interest and Funding

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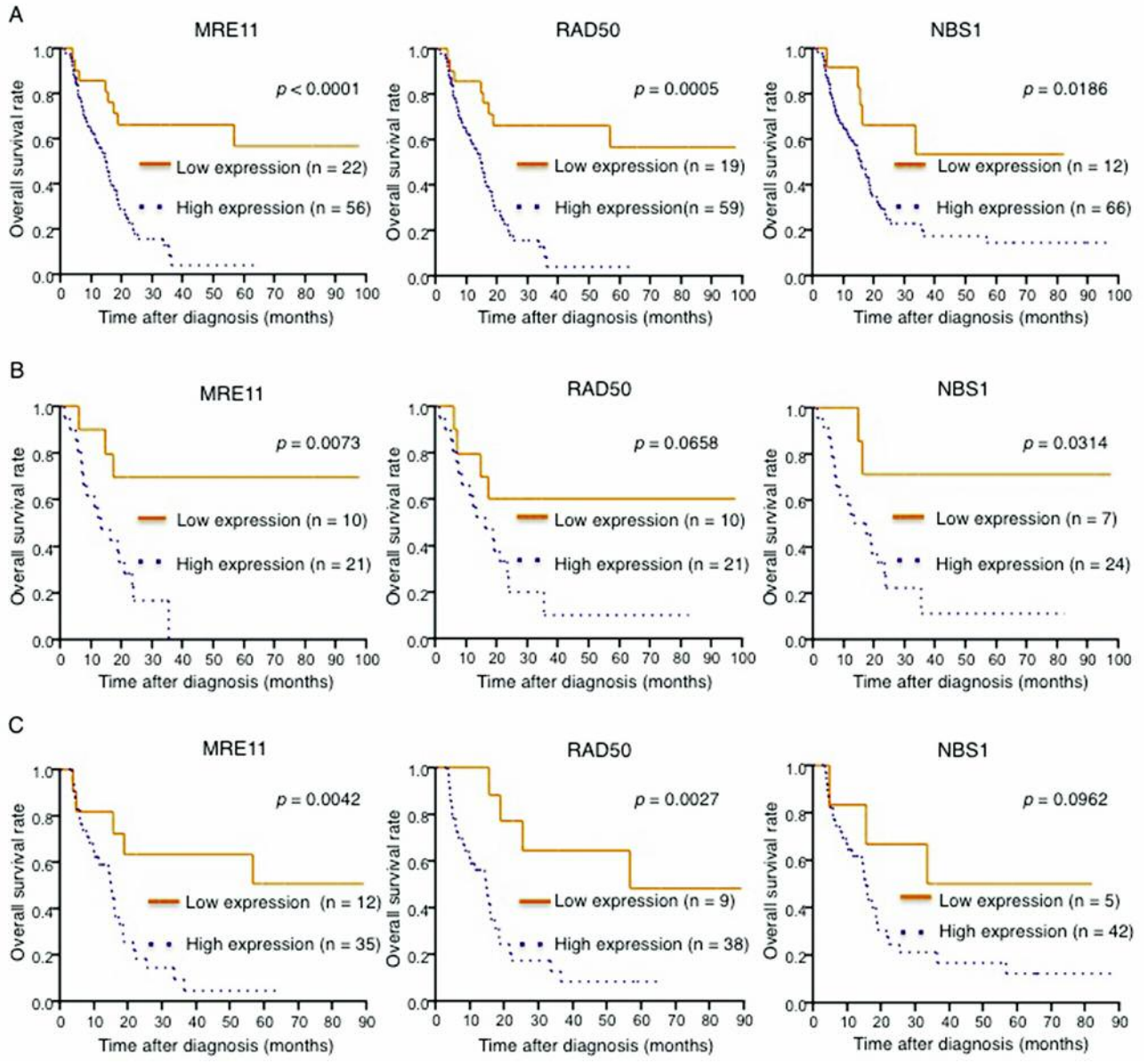

Figure 4. *Correlation of overall survival with the expression of nuclear meiotic recombination 11 (MRE11), DNA repair protein Rad50 (RAD50) and Nijmegen breakage syndrome 1 (NBS1) complex in 78 biopsy samples before chemotherapy from patients with unresectable gastric cancer (GC) at initial diagnosis. A: Overall survival rate according to nuclear expression of MRE11, RAD50, and NBS1. B: Overall survival rate of 31 patients with unresectable GC at initial diagnosis treated by cisplatin plus TS-1 as first-line regimen according to nuclear expression of MRE11, RAD50, and NBS1. C: Overall survival rate of 47 patients with unresectable GC at initial diagnosis treated by paclitaxel plus TS-1 as first-line regimen according to nuclear expression of MRE11, RAD50, and NBS1.*

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Table IV. *Univariate and multivariate analyses of clinicopathological factors according to the Japanese Classification of Gastric Carcinoma affecting overall survival of patients with unresectable gastric cancer. Protein expression was determined from biopsy specimens.*

Clinicopathological variable	Univariate			Multivariate (MRE11)			Multivariate (RAD50)			Multivariate (NBS1)		
	RR	95% CI	*p*-Value	RR	95% CI	*p*-Value	RR	95% CI	*p*-Value	RR	95% CI	*p*-Value
Age: <65/≥65 years	1.06	0.62-1.8	0.844	-	-	-	-	-	-	-	-	-
Gender: Male/female	1.23	0.69-2.27	0.4811	-	-	-	-	-	-	-	-	-
Histology grade: Differentiated/undifferentiated	1.66	0.95-3.00	0.0718	-	-	-	-	-	-	-	-	-
Tumor depth: SS, SE/SI	0.72	0.31-1.45	0.3786	-	-	-	-	-	-	-	-	-
Lymph node metastasis: Absent/present	0.52	0.24-1.37	0.1719	-	-	-	-	-	-	-	-	-
Hepatic metastases: Absent/present	1.5	0.68-2.95	0.293	-	-	-	-	-	-	-	-	-
Distant metastases: Absent/present	1.67	0.83-3.10	0.1418	-	-	-	-	-	-	-	-	-
Peritoneal metastases: Absent/present	1.58	0.91-2.70	0.1028	-	-	-	-	-	-	-	-	-
Stage: III/IV	2.62	1.14-7.59	0.0206*	2.4	1.04-6.96	0.0384*	2.81	1.84-8.64	0.0127*	2.52	1.09-7.32	0.290
MRE11 expression: Low/high	4.37	2.12-10.28	<0.0001*	4.18	2.03-9.80	<0.0001*	-	-	-	-	-	-
RAD50 expression: Low/high	3.59	1.77-8.28	0.0002*	-	-	-	3.73	1.22-8.17	<0.0001*	-	-	-
NBS1 expression: Low/high	2.88	1.28-8.34	0.0097*	-	-	-	-	-	-	2.78	1.21-8.04	0.0135*

MRE11: Meiotic recombination 11; RAD50: DNA-repair protein Rad50; NBS1: Nijmegen breakage syndrome 1; SS: subserosal invasion; SE: serosal invasion; SI: surrounding organ invasion; RR: relative risk; CI: confidence interval. *Significant result.

Table V. *Multivariate analyses of existing factors affecting resectability and meiotic recombination 11 (MRE11), DNA-repair protein Rad50 (RAD50) and Nijmegen breakage syndrome 1 (NBS1) affecting surgery after down-staging by chemotherapy for unresectable gastric cancer.*

Clinicopathological variable	MRE11			RAD50			NBS1		
	OR	95% CI	*p*-Value	OR	95% CI	*p*-Value	OR	95% CI	*p*-Value
Tumor depth: SS,SE/SI	1.53	0.29-9.74	0.6197	1.36	0.27-8.43	0.7168	2.13	0.50-11.58	0.3137
Hepatic metastases: Absent/present	7.03E+07	3.67-‡	0.003*	8.00E+08	4.16-‡	0.0019*	1.97E+07	5.644E+22-‡	0.0025*
Peritoneal metastases: Absent/present	2.53	0.66-11.07	0.1756	2.66	0.72-11.34	0.1434	2.31	0.77-7.44	0.1386
MRE11expression: Low/high	21.17	5.83-94.83	<0.0001*	-	-	-	-	-	-
RAD50 expression: Low/high	-	-	-	17.98	4.78-84.25	<0.0001*	-	-	-
NBS1 expression: Low/high	-	-	-	-	-	-	3.41	0.86-14.43	0.0806

OR, Odds ratio of avoiding surgery to having to undergo surgery; CI, confidence interval; SS: subserosal invasion; SE: serosal invasion; SI: surrounding organ invasion. *Significant result. ‡Upper confidence intervals were not calculated.

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