DNA Damage Response-Related Proteins in Gastric Cancer: ATM, Chk2 and p53 Expression and Their Prognostic Value

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Key Words

Stomach neoplasm · DNA damage response · Ataxia telangiectasia mutated protein · Checkpoint kinase 2 · Tumor suppressor protein p53 · Survival analysis · Immunohistochemistry

Abstract

Objectives: The aims of this study were to assess expressions of the DNA damage response (DDR)-related proteins and to investigate their clinical significances in gastric carcinoma.

Methods: Two independent cohorts, a training set (n = 524) and validation set (n = 394), of gastric cancer patients were enrolled. Ataxia telangiectasia mutated (ATM), checkpoint kinase 2 (Chk2), and p53 expressions were examined by immunohistochemistry using tissue microarray. **Results:** ATM loss, Chk2 loss, and p53 positivity were observed in 21.8, 14.1, and 36.1% of the training set, and in 17.3, 12.2, and 35.8% of the validation set, respectively. In the training set, the aberrant expressions of ATM, Chk2, or p53 were significantly associated with an advanced TNM stage and poor disease-specific survival. This association was verified in the validation set. Chk2 positivity and p53 negativity were significantly related to a prolonged disease-specific survival. Also, patients with nonaberrant expressional levels of all 3 DDR-

related proteins had a more favorable outcome than others. Multivariate analyses showed that Chk2 loss and at least 1 aberrant DDR-related protein remained as independent prognostic factors of poor disease-specific survival. **Conclusions:** This study elucidated the prognostic implications of DDR-related proteins, and suggests that their aberrant expressions play critical roles in the development and progression of gastric cancer.

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Introduction

Gastric cancer has been reported to be the fourth most common cancer and the second leading cause of cancer-related death worldwide [1]. More than 930,000 new cases are diagnosed and 700,000 deaths are attributed to gastric cancer annually [2]. At present, surgical resection is the mainstay of treatment, but even after potentially curative resection, the 5-year survival rate is only around 40% [3–5].

Tumor-node-metastasis (TNM) stage (by UICC/AJCC) and completeness of surgical excision are considered to be the most powerful prognostic factors in gastric cancer [6]. However, it is not uncommon that gastric can-

cer patients with the same TNM stage pursue quite different clinical courses, and despite continued efforts to identify markers of aggressive disease, no clinically meaningful marker has yet been identified. Thus, the search continues to find prognostic factors of survival, and DNA damage response (DDR)-related proteins are obvious candidate proteins.

When DNA damage occurs, DDR is activated, which includes activation of a cell cycle checkpoint, the initiation of transcriptional programs, and the execution of DNA repair or initiation of apoptosis. The term 'cell cycle checkpoint' refers to control mechanisms that ensure the fidelity of cell division in eukaryotic cells by monitoring the successful completion of upstream events prior to proceeding to the next phase [7]. Defects in DDRs or in mitotic-checkpoint pathways can permit the survival or the continued growth of cells with genomic damage, thereby enhancing the likelihood of malignant transformation [7]. As a result, the cell cycle checkpoint is considered as a strong candidate of the anticancer barrier [8]. The key regulators of checkpoint pathways in mammalian DDR are the ataxia telangiectasia mutated (ATM) and Rad3-related proteins. In particular, ATM, a serine/threonine protein kinase, senses double-strand damage and subsequently activates the DNA damage checkpoint, which leads to cell cycle arrest through the ATM/checkpoint kinase 2 (Chk2)-p53/mouse double minute 2 homolog-p21 pathways [7, 9]. These findings led us to conjecture that the aberrant expressions of DDR-related proteins, such as ATM, Chk2, and p53, might allow tumorigenic cells to evade normal cell cycle checkpoints, and cause aberrant cell proliferation and survival, increased genomic instability, and ultimately tumor progression. Accordingly, we hypothesized that DDR-related protein alterations might influence tumor progression, and be associated with patient survival in gastric cancer.

In the present study, we evaluated the expressions of ATM, Chk2, and p53 in human gastric carcinoma tissues obtained from two independent cohorts using immunohistochemistry, and followed this with an analysis of associations between their expressions and clinicopathological parameters or patient survival. Also, we hypothesized that the negative regulation of cell proliferation due to DNA damage would not function in tumors with a DDR-related protein alteration. It is well known that Ki-67 is a cell growth-related antigen [10–12], and thus, we used a Ki-67 labeling index as a surrogate of cell proliferative status. Accordingly, we sought to determine whether expression alterations in cell cycle checkpoint-related proteins are related with a Ki-67 proliferation index.

Materials and Methods

Patients

The files of 524 surgically resected primary gastric carcinoma cases examined at the Department of Pathology, Seoul National University Hospital (Seoul), for the whole of 1995 (training set) [13] and the files of 394 surgically resected primary gastric carcinoma cases examined at the Department of Pathology, Seoul National University Hospital, for the whole of 2004 (validation set) [14] were analyzed. Age, gender, tumor location, WHO and Lauren's classification, pTNM (pathological TNM) stage (by the 7th UICC/AJCC manual) [1], lymphatic invasion, lymph node metastasis, and distant metastasis were evaluated by reviewing medical charts, pathological records, gross photographs, and glass slides. Median follow-up periods of patients in the training set and the validation set were 97.0 months (range, 1–108) and 49.4 months (range, 0–60), respectively. The cases lost to follow-up and deaths from any other cause other than gastric cancer were regarded as censored data for the analysis of survival rates. No patient received preoperative chemoradiotherapy, and patients with stage II, III, or IV disease received postoperative chemotherapy using a 5-fluorouracil-based regimen (alone, plus mitomycin C, or plus cisplatin). The study protocol was reviewed and approved by the Institutional Review Board of Seoul National University Hospital.

Tissue Array Methods

Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded gastric carcinomas (donor blocks) and arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus (Superbiochips Laboratories, Seoul). Because we have previously shown excellent agreement between the staining results of different intratumoral areas in gastric carcinoma [13], a core was sampled from each case. An adequate case was defined as a tumor occupying more than 10% of the core area. Sections (4 µm) were cut from each tissue array block, deparaffinized, and dehydrated.

Immunohistochemistry

Immunohistochemical staining was performed using an automatic immunostainer (Bond-Max, Leica Microsystems, Wetzlar, Germany) according to the manufacturer's instructions. The primary antibodies used were as follows: anti-ATM (Y170; rabbit monoclonal; 1:50; Epitomics, Burlingame, Calif., USA), anti-Chk2 (IMG-336; mouse monoclonal; 1:300; Imgenex, San Diego, Calif., USA), anti-p53 (M7001; mouse monoclonal; 1:200; Dako, Carpinteria, Calif., USA), and anti-Ki-67 (MIB-1; mouse monoclonal; 1:100; Dako). Cancer cells showing nuclear staining, regardless of the presence of cytoplasmic staining, were considered positively immunostained for ATM, Chk2, p53, and Ki-67. Reduction or lack of staining was restricted to tumor cells; the stromal elements surrounding tumor nests preserved the normal staining pattern, thereby providing an internal control on the same section. Immunostaining of ATM, Chk2, and p53 was scored in a semiquantitative manner considering the proportion of positive cells using a scale of 0–4: 0, no staining; 1, positive in less than or equal to 10%; 2, positive in more than 10%, but less than or equal to 33%; 3, positive in more than 33%, but less than or equal to 66%, and 4, positive in more than 66%. For Ki-67, areas of highest proliferative activity were searched by scanning tumor sections at low magnification due to the heterogeneous distribution of proliferative tumor

cells. Tumor cell counting was then performed within these areas at a magnification of $\times 200$. One hundred tumor cells were counted per tumor. The Ki-67 proliferation index was defined as the number of tumor cell nuclei expressing MIB-1 expressed as a percentage of total tumor cell nuclei.

Statistical Analyses

The survival curves were plotted using the Kaplan-Meier product-limit method, and significance of difference between survival curves was determined using the log-rank test. Multivariate survival analysis was performed using the Cox proportional hazards model. The χ^2 test or Fisher's exact test (2-sided) was used to determine the nature of the correlations between antibody expressions and clinicopathological parameters. The relationship between the expression of each DDR protein and cell proliferation index was analyzed using the 2-tailed Student's *t* test. Results were considered significant when *p* values were < 0.05 . All statistical analyses were conducted using the PASW18.0 statistical software program (SPSS, Chicago, Ill., USA).

Results

DDR-Related Protein Expressions

The expression levels of ATM, Chk2, and p53 were determined by immunohistochemistry. Representative results of immunohistochemical staining are shown in figure 1. We applied the 'minimum *p* value' approach to obtain the cutoff value for each antibody providing the best separation between the groups of patients related to disease-specific survival in the training set. According to this approach, a score of 0 or 1 were regarded as negative while 2–4 as positive for all three antibodies, and the cutoffs were also applied to the validation set. As a result, in the training set including 524 cases, 114 (21.8%), 74 (14.1%), and 189 (36.1%) showed loss of ATM, loss of Chk2, and p53 positivity, respectively. Also, in a total of 394 cases from the validation set, loss of ATM, loss of Chk2, and p53 positivity were observed in 68 (17.3%), 48 (12.2%), and 141 (35.8%) cases, respectively. When cases were grouped into 'DDR-related protein intact' (DDRRP-I; no aberrant expression of ATM, Chk2, or p53) and 'DDR-related protein aberrant' (DDRRP-A; aberrant expression of at least 1 of the 3 proteins), 236 (45.0%) cases in the training set and 184 (46.7%) in the validation set were categorized as DDRRP-I, and 288 (55.0%) cases in the training set and 210 (53.3%) in the validation set as DDRRP-A.

We analyzed the associations among the aberrant expressions of each DDR-related protein, and found that Chk2 loss and ATM loss were significantly correlated ($p < 0.001$, both in the training set and in the validation set) and patients with p53 negativity tended to have Chk2

loss rather than the intact expression in the validation set ($p = 0.047$). On the other hand, the aberrant expressions of p53 and ATM in both sets and those of p53 and Chk2 in the training set were not significantly related.

Associations between the Expressions of DDR-Related Proteins and the Ki-67 Proliferation Index

We supposed that damage to DDR-related proteins might cause tumor cell proliferation. Thus, we measured Ki-67 proliferation indices, and sought relations between them and DDR-related protein expressions in the validation set. As was expected, the Ki-67 proliferation index was significantly higher in the p53-positive group than in the p53-negative group (36 ± 20 vs. $26 \pm 22\%$; $p < 0.001$). On the other hand, no statistically meaningful relationships were found between Ki-67 proliferation index and ATM or Chk2 expression. When performing the combination analysis, the DDRRP-A group was found to have a significantly higher Ki-67 proliferation index than the DDRRP-I group (33 ± 21 vs. $26 \pm 22\%$; $p = 0.001$).

Associations between the Expressions of DDR-Related Proteins and Clinicopathological Parameters

The correlations found between the expressions of ATM, Chk2, and p53 and clinicopathological features of the gastric cancer specimens are summarized in tables 1 and 2. In the training set, ATM negativity ($p < 0.001$), Chk2 negativity ($p = 0.004$), p53 positivity ($p = 0.018$), or DDRRP-A ($p < 0.001$) were significantly correlated with a higher TNM stage. In the validation set, ATM negativity ($p = 0.018$), p53 positivity ($p < 0.001$), or DDRRP-A ($p < 0.001$) was also found to be significantly associated with a higher TNM stage.

Associations between DDR-Related Protein Expressions and Patient Survival

In order to determine the natures of correlations between survival rate and the expressions of ATM, Chk2, and p53, we used a log-rank test with Kaplan-Meier estimates. Of the 524 patients from the training set, those with ATM positivity ($p < 0.001$), Chk2 positivity ($p < 0.001$), p53 negativity ($p = 0.003$), or DDRRP-I ($p < 0.001$) had significantly longer survival times than those with ATM negativity, Chk2 negativity, p53 positivity, or DDRRP-A, respectively (fig. 2). Multivariate Cox hazard models revealed that Chk2 negativity and DDRRP-A were independent biomarkers of poor disease-specific survival (hazard ratio = 1.390, 95% confidence interval, CI = 1.003–1.927, and 1.366, 95% CI = 1.029–1.813, respectively; table 3), after adjusting for the following co-

Fig. 1. DDR-related protein expressions in gastric cancer. Immunohistochemical staining for ATM (**a, b**), Chk2 (**c, d**), and p53 (**e, f**) is shown. Altered expressions of these proteins, loss of ATM (**b**), loss of Chk2 (**d**), and over-expression of p53 (**e**), were observed in 9.9, 12.2, and 35.8% of the gastric carcinoma cases, respectively. Original magnification $\times 200$.

variates: tumor invasion, lymph node metastasis, and distant metastasis, which were all significant prognostic factors by univariate analyses. However, ATM and p53 were not independent prognostic indicators.

Because prognosis and treatment strategy for patients with TNM stage IV are quite different from those for pa-

tients with TNM stages I–III, we divided the patients into groups of stages I–III ($n = 481$) and of stage IV ($n = 43$), and performed subgroup analyses. Within the subgroup of stages I–III, ATM positivity ($p = 0.002$), Chk2 positivity ($p = 0.001$), p53 negativity ($p = 0.003$), or DDRRP-I ($p < 0.001$) was significantly associated with a prolonged

Table 1. Correlations between expressions of ATM, Chk2, or p53 and clinicopathological parameters in gastric carcinomas in the training set

	ATM		Chk2		p53		DDR-related protein	
	negative (n = 114)	positive (n = 410)	negative (n = 74)	positive (n = 450)	negative (n = 335)	positive (n = 189)	DDRRP-I (n = 236)	DDRRP-A (n = 288)
Mean age $\pm$ SD, years	56.0 $\pm$ 12.2	54.8 $\pm$ 12.8	56.4 $\pm$ 12.4	54.9 $\pm$ 12.7	53.8 $\pm$ 12.5	57.3 $\pm$ 12.7	52.9 $\pm$ 12.7	56.8 $\pm$ 12.4
Gender								
Male	60 (17)	286 (83)	42 (12)	304 (88)	214 (62)	132 (38)	164 (47)	182 (53)
Female	54 (30)	124 (70)	32 (18)	146 (82)	121 (68)	57 (32)	72 (40)	106 (60)
WHO classification								
WD	4 (11)	33 (89)	2 (5)	35 (95)	21 (57)	16 (43)	19 (51)	18 (49)
MD	34 (22)	119 (78)	23 (15)	130 (80)	93 (61)	60 (39)	64 (42)	89 (58)
PD	55 (23)	182 (77)	35 (15)	202 (85)	146 (62)	91 (38)	100 (42)	137 (58)
Mucinous	14 (40)	21 (60)	7 (20)	28 (80)	27 (77)	8 (23)	13 (37)	22 (63)
SRC	7 (11)	54 (89)	7 (11)	54 (89)	48 (79)	13 (21)	40 (66)	21 (34)
Others	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)
Lauren classification								
Intestinal	41 (22)	149 (78)	25 (13)	165 (87)	114 (60)	76 (40)	80 (42)	110 (58)
Diffuse	65 (22)	227 (78)	40 (14)	252 (86)	190 (65)	102 (35)	133 (45)	159 (55)
Mixed	8 (19)	34 (81)	9 (21)	33 (79)	31 (74)	11 (26)	23 (55)	19 (55)
Tumor invasion								
pT1	9 (6)	137 (94)	10 (7)	136 (93)	107 (73)	39 (27)	96 (66)	50 (34)
pT2	9 (18)	42 (82)	3 (6)	48 (94)	29 (57)	22 (43)	24 (47)	27 (53)
pT3	67 (31)	147 (69)	34 (16)	180 (84)	133 (62)	81 (38)	76 (36)	138 (64)
pT4	29 (26)	84 (74)	27 (24)	86 (76)	66 (58)	47 (42)	40 (35)	73 (65)
Lymph node metastasis								
Absent	28 (16)	152 (84)	14 (8)	166 (92)	133 (74)	47 (26)	107 (59)	73 (41)
Present	86 (25)	258 (75)	60 (17)	284 (83)	202 (59)	142 (41)	129 (37)	215 (63)
Distant metastasis								
Absent	98 (21)	377 (79)	64 (13)	411 (87)	308 (65)	167 (35)	221 (46)	254 (54)
Present	15 (35)	28 (65)	10 (23)	33 (77)	24 (56)	19 (44)	13 (30)	30 (70)
pTNM stage								
I	12 (8)	146 (92)	10 (6)	148 (94)	115 (73)	43 (27)	103 (65)	55 (35)
II	37 (32)	77 (68)	17 (15)	97 (85)	75 (66)	39 (34)	43 (38)	71 (62)
III	50 (24)	159 (76)	37 (18)	172 (82)	121 (58)	88 (42)	77 (37)	132 (63)
IV	15 (35)	28 (65)	10 (23)	33 (77)	24 (56)	19 (44)	13 (30)	30 (70)
Lymphatic invasion								
Absent	69 (19)	289 (81)	44 (12)	314 (88)	237 (66)	121 (34)	175 (49)	183 (51)
Present	45 (27)	121 (73)	30 (18)	136 (82)	98 (59)	189 (36)	61 (37)	105 (63)
Vascular invasion								
Absent	107 (21)	395 (79)	70 (14)	432 (86)	323 (64)	179 (36)	229 (46)	273 (54)
Present	7 (32)	15 (68)	4 (18)	18 (82)	12 (55)	10 (45)	7 (32)	15 (68)

χ^2 or Fisher's exact test was used in each analysis except for the analysis of the age factor (Student's t test); data with p values of statistically significant correlations are indicated in italics; values in parentheses are percentages; SD = standard deviation; WD = well-differentiated tubular adenocarcinoma; MD = moderately differentiated tubular adenocarcinoma; PD = poorly differentiated tubular adenocarcinoma; mucinous = mucinous adenocarcinoma; SRC = signet ring cell carcinoma; pTNM = pathological tumor-node-metastasis.

survival. In the subgroup of stage IV, on the other hand, the survival time was not different according to the expressional status of ATM, Chk2, or p53 expression status.

In the validation set including 394 patients, patients with Chk2 positivity ($p = 0.001$), p53 negativity ($p < 0.001$), or DDRRP-I ($p < 0.001$) showed significantly lon-

ger survival times than those with Chk2 negativity, p53 positivity, or DDRRP-A, respectively. Patients with ATM positivity tended to have longer survival times, although it was not statistically significant ($p = 0.189$; fig. 3). Multivariate Cox hazard models revealed that Chk2 negativity and DDRRP-A were independent biomarkers of poor

Table 2. Correlations between expressions of ATM, Chk2, or p53 and clinicopathological parameters in gastric carcinomas in the validation set

	ATM		Chk2		p53		DDR-related protein	
	negative (n = 68)	positive (n = 326)	negative (n = 48)	positive (n = 346)	negative (n = 253)	positive (n = 141)	DDRRP-I (n = 184)	DDRRP-A (n = 210)
Mean age $\pm$ SD, years	62.4 $\pm$ 10.8	56.5 $\pm$ 12.9	56.6 $\pm$ 11.5	57.6 $\pm$ 13.0	56.2 $\pm$ 12.4	59.8 $\pm$ 13.1	55.0 $\pm$ 12.5	60.0 $\pm$ 12.5
Gender								
Male	46 (16)	237 (84)	32 (11)	251 (89)	174 (62)	109 (38)	132 (47)	151 (53)
Female	22 (20)	89 (80)	16 (14)	95 (86)	79 (71)	32 (29)	52 (47)	59 (53)
WHO classification								
WD	3 (12)	22 (88)	0 (0)	25 (100)	20 (80)	5 (20)	17 (68)	8 (32)
MD	30 (22)	106 (78)	21 (15)	115 (85)	71 (52)	65 (48)	41 (30)	95 (70)
PD	20 (14)	124 (86)	15 (10)	129 (90)	94 (65)	50 (35)	73 (51)	71 (49)
Mucinous	7 (50)	7 (50)	3 (21)	11 (79)	9 (64)	5 (36)	3 (21)	11 (79)
SRC	6 (9)	61 (91)	8 (12)	59 (88)	56 (84)	11 (16)	48 (72)	19 (28)
Others	2 (25)	6 (75)	1 (12)	7 (88)	3 (38)	5 (63)	2 (25)	6 (75)
Lauren classification								
Intestinal	34 (21)	126 (79)	24 (15)	136 (85)	93 (58)	67 (42)	60 (38)	100 (62)
Diffuse	19 (12)	141 (88)	19 (12)	141 (88)	113 (71)	47 (29)	91 (57)	69 (43)
Mixed	15 (21)	57 (79)	5 (7)	67 (93)	47 (65)	25 (35)	33 (46)	39 (42)
Undetermined	0 (0)	2 (100)	0 (0)	2 (100)	0 (0)	2 (100)	0 (0)	2 (100)
Tumor invasion								
pT1	10 (10)	94 (90)	5 (5)	99 (95)	80 (77)	24 (23)	68 (65)	36 (35)
pT2	14 (23)	47 (77)	10 (16)	51 (84)	48 (79)	13 (21)	33 (54)	28 (46)
pT3	27 (19)	114 (81)	22 (16)	119 (84)	81 (57)	60 (43)	55 (39)	86 (61)
pT4	17 (19)	71 (81)	11 (13)	77 (87)	44 (50)	44 (50)	28 (50)	60 (50)
Lymph node metastasis								
Absent	20 (13)	140 (87)	16 (10)	144 (90)	123 (77)	37 (23)	94 (59)	66 (41)
Present	48 (21)	186 (79)	32 (14)	202 (86)	130 (56)	104 (44)	90 (39)	144 (61)
Distant metastasis								
Absent	56 (16)	287 (84)	38 (11)	305 (89)	227 (66)	116 (34)	169 (49)	174 (51)
Present	12 (24)	39 (76)	10 (20)	41 (80)	26 (51)	25 (49)	15 (29)	36 (71)
pTNM stage								
I	16 (12)	113 (88)	11 (9)	118 (91)	102 (79)	27 (21)	80 (62)	49 (38)
II	10 (11)	80 (89)	9 (10)	81 (90)	64 (71)	26 (29)	51 (57)	39 (43)
III	30 (24)	94 (76)	18 (14)	106 (86)	61 (49)	63 (51)	38 (31)	86 (69)
IV	12 (24)	39 (76)	10 (20)	41 (80)	26 (51)	25 (49)	15 (29)	36 (71)
Lymphatic invasion								
Absent	18 (12)	138 (89)	13 (8)	143 (92)	100 (77)	36 (23)	97 (62)	59 (38)
Present	50 (21)	188 (79)	35 (15)	203 (85)	133 (56)	105 (44)	87 (37)	151 (63)
Vascular invasion								
Absent	50 (16)	271 (84)	36 (11)	285 (89)	218 (68)	103 (32)	162 (51)	159 (49)
Present	18 (25)	55 (75)	12 (16)	61 (84)	35 (48)	38 (52)	22 (30)	51 (70)

χ^2 or Fisher's exact test was used in each analysis except for the analysis of the age factor (Student's *t* test); data with *p* values of statistically significant correlations are indicated in italics; values in parentheses are percentages; SD = standard deviation; WD = well-differentiated tubular adenocarcinoma; MD = moderately differentiated tubular adenocarcinoma; PD = poorly differentiated tubular adenocarcinoma; mucinous = mucinous adenocarcinoma; SRC = signet ring cell carcinoma; pTNM = pathological tumor-node-metastasis.

survival, after adjusting for tumor invasion, lymph node metastasis, and distant metastasis (hazard ratio = 1.970, 95% CI = 1.245–3.116, and 1.524, 95% CI = 1.029–2.257, respectively; table 3). On the other hand, p53 was not found to be an independent prognostic indicator. Within the subgroup of stages I–III (*n* = 343), Chk2 positivity

(*p* = 0.043), p53 negativity (*p* < 0.001), or DDRRP-I (*p* < 0.001) was significantly associated with better survival, but the ATM expression status was not related to patient survival. In the subgroup of stage IV (*n* = 51), patients with Chk2 positivity (*p* = 0.005) had longer survival times than those with Chk2 negativity, but the expres-

Fig. 2. Kaplan-Meier survival plots for the 524 gastric cancer patients from the training set as a function of DDR-related protein expressions. Patients with ATM negativity (**a**), Chk2 negativity (**b**), or p53 positivity (**c**) showed a significantly shorter survival

time than those with ATM positivity, Chk2 positivity, or p53 negativity, respectively ($p < 0.001$, $p < 0.001$, and $p = 0.003$). Also, patients with all 3 DDRRP-I had a significantly longer survival time than those with at least 1 DDRRP-A ($p < 0.001$; **d**).

sional status of ATM and p53 and combination of the DDR-related protein expression were not associated with patient survival.

Discussion

In the present study, we determined the expressional status of the DDR-related proteins ATM, Chk2, and p53 in gastric cancer, and correlations between their expressional status and proliferation index, clinicopathological parameters, and patient survival. Alterations in the expressions of DDR-related proteins were found to be associated with an advanced TNM stage and poor prognosis. Specifically, Chk2 loss or the aberrant expression of one or more DDR-related proteins (ATM, Chk2, and

p53) were found to be independent prognostic indicators of poor survival, even after adjusting for other prognostic factors. In addition, we found that the aberrant expression of DDR-related proteins is correlated with a high Ki-67 proliferation index, as was initially expected. To the best of our knowledge, this is the first report to describe the expressional patterns of proteins belonging to the ATM-Chk2-p53 pathway thoroughly, to describe the associations between their expressions and clinicopathological parameters, and to determine their prognostic roles in the large human gastric cancer series. Furthermore, the large number of study targets (918 patients), the validation of the data in an independent cohort (validation set), and the consistency of the results of two independent cohorts (training set and validation set), all increase the reliability of the findings of this study.

Table 3. Multivariate Cox proportional hazard models for the predictors of disease-specific survival

Variables	Hazard ratio	p value
<i>Training set</i>		
Model 1		
ATM expression (positive vs. negative)	1.301 (0.971–1.744)	0.078
Tumor invasion (EGC vs. AGC)	4.953 (2.348–8.985)	<0.001
Lymph node metastasis (no vs. yes)	2.811 (1.762–4.485)	<0.001
Distant metastasis (no vs. yes)	3.473 (2.420–4.984)	<0.001
Model 2		
Chk2 expression (positive vs. negative)	1.390 (1.003–1.927)	0.048
Tumor invasion (EGC vs. AGC)	4.852 (2.491–9.454)	<0.001
Lymph node metastasis (no vs. yes)	2.691 (1.687–4.293)	<0.001
Distant metastasis (no vs. yes)	3.452 (2.405–4.953)	<0.001
Model 3		
p53 expression (negative vs. positive)	1.169 (0.893–1.532)	0.257
Tumor invasion (EGC vs. AGC)	4.899 (2.514–9.549)	<0.001
Lymph node metastasis (no vs. yes)	2.685 (1.680–4.292)	<0.001
Distant metastasis (no vs. yes)	3.471 (2.418–4.981)	<0.001
Model 4		
DDR-related proteins (DDRRP-I vs. DDRRP-A)	1.366 (1.029–1.813)	0.031
Tumor invasion (EGC vs. AGC)	4.622 (2.369–9.018)	<0.001
Lymph node metastasis (no vs. yes)	2.683 (1.682–4.279)	<0.001
Distant metastasis (no vs. yes)	3.479 (2.425–4.993)	<0.001
<i>Validation set</i>		
Model 1		
Chk2 expression (positive vs. negative)	1.970 (1.245–3.116)	0.004
Tumor invasion (EGC vs. AGC)	8.775 (2.122–36.288)	0.003
Lymph node metastasis (no vs. yes)	6.250 (2.854–13.689)	<0.001
Distant metastasis (no vs. yes)	5.601 (3.794–8.268)	<0.001
Model 2		
p53 expression (negative vs. positive)	1.280 (0.890–1.840)	0.183
Tumor invasion (EGC vs. AGC)	9.060 (2.186–37.538)	0.002
Lymph node metastasis (no vs. yes)	5.869 (2.677–12.866)	<0.001
Distant metastasis (no vs. yes)	5.257 (3.564–7.753)	<0.001
Model 3		
DDR-related proteins (DDRRP-I vs. DDRRP-A)	1.524 (1.029–2.257)	0.036
Tumor invasion (EGC vs. AGC)	8.434 (2.030–35.047)	0.003
Lymph node metastasis (no vs. yes)	5.902 (2.695–12.925)	<0.001
Distant metastasis (no vs. yes)	5.325 (3.618–7.837)	<0.001

95% confidence intervals are indicated in parentheses; EGC = early gastric carcinoma; AGC = advanced gastric carcinoma.

It is evident that although several proteins participate in DDR in a stepwise fashion, they influence by not only one way. In the present study, our combination analysis of the 3 DDR-related proteins suggested that even the aberrant expression of only 1 of the 3 proteins causes an unfavorable clinical course. However, the biological links between genetic alterations in these proteins remain unclear. Further study is needed to reveal the links.

It has been thought that DDR-related proteins were related to chemosensitivity, and as a result, inhibiting these proteins was believed to sensitize tumor cells to can-

cer treatments that damage DNA [15, 16]. Because the patients with gastric cancer in the present study were treated with a chemotherapy regimen containing DNA-damaging agents postoperatively, the expressional status of DDR-related proteins possibly affected the chemore-sponse of the tumor. Furthermore, as the prognosis of stage IV patients is more influenced by the chemore-sponse, the aberrant expression of the DDR-related proteins could exert an effect toward a favorable clinical outcome through the chemosensitive property. That may be a part of the reason that the expressional status of the

Fig. 3. Kaplan-Meier survival plots for the 394 gastric cancer patients from the validation set as a function of DDR-related protein expressions. ATM expression status was not significantly associated with patient survival (**a**). Patients with Chk2 negativity (**b**) or p53

positivity (**c**) showed a significantly shorter survival time than those with Chk2 positivity or p53 negativity, respectively ($p = 0.001$ and $p < 0.001$). Also, patients with all 3 DDRRP-I had a significantly longer survival time than those with at least 1 DDRRP-A ($p < 0.001$; **d**).

DDR-related proteins did not affect patient survival in the subgroup of stage IV patients, whereas their aberrant expressions were significantly related to poor survival in the subgroup of stages I–III. Further study will be needed to elucidate it.

Due to the important role played by ATM in the control of genomic stability, we surmise that defective ATM is likely to be associated with a high cancer risk and a poor prognosis. Furthermore, recent studies have indicated associations between promoter hypermethylation of the *ATM* gene and reduced postoperative survival in head and neck squamous cell carcinoma, non-small cell lung cancer, and breast cancer [17], and another study reported that the expressional loss of ATM was associated with poor prognosis in colorectal cancer [18]. In ovarian cancer, although ATM and Chk2 protein expressions were not found to be individually related to overall survival, at

least one alteration was found to be a negative predictor of overall survival [19]. On the other hand, the expressions of ATM and Chk2 were not found to have predictive value in multimodality-treated esophageal squamous cell carcinoma [20]. With regard to gastric cancer, it has been reported that the presence of a low level of phosphorylated ATM is significantly correlated with poor differentiation, lymph node metastasis, and poor 5-year survival [21]. However, in the present study, no significant survival difference was observed between the ATM-positive and -negative groups, although the ATM-positive group tended to have a longer survival time. This discrepancy between the results may be due to the use of ATM or phosphorylated ATM and to different levels of histological differentiation and TNM stage.

Studies of Li-Fraumeni syndrome have identified germline mutations in the *TP53* or *CHEK2* genes [22, 23].

Mutated Chk2 protein may be functionally defective in terms of its protein-protein interactions and phosphorylation by ATM [23]. Furthermore, it has been reported that patients with Li-Fraumeni syndrome are predisposed to breast cancer, sarcoma, brain carcinoma, and adrenal cortex carcinoma, and that sometimes multiple tumors are identified in the same individual [24]. In addition, it has been reported that cases with high Chk2 protein or mRNA expression tend to have mutated *TP53*, and in the same study, no relation was found between Chk2 expression and histological differentiation or depth of tumor invasion in gastric cancer [25]. However, to date, no study has revealed any correlation between Chk2 expression and survival in gastric cancer.

Many contradictory results have been reported regarding the prognostic role of p53 in gastric cancer, and whereas some have concluded that aberrant p53 expression is associated with poor survival, others have found no relation [26]. In the present study, patients with aberrant p53 expression were found to have significantly shorter survival times than those with intact p53 expression by the log-rank test. However, p53 expression was not found to be an independent prognostic factor by multivariate analysis.

Although we had shown a good agreement of protein expressions among different tissue cores of each tumor in the previous study [13], intratumoral genetic heterogeneity has become an important issue in cancer therapeutics. Recently, Gerlinger et al. [27] demonstrated extensive intratumoral heterogeneity by exome sequencing, chromosome aberration analysis, and ploidy profiling on multiple spatially separated samples obtained from primary renal carcinomas and associated metastatic sites. Also, they insisted that intratumoral heterogeneity can lead to underestimation of the tumor genomics landscape

portrayed from single tumor biopsy samples and may present major challenges to personalized-medicine and biomarker development [27]. Therefore, in order that the DDR-related proteins of our study are clinically used as tumor biomarkers, further investigation regarding intratumoral heterogeneity would be required.

Recently, it was indicated that ATM deficiency sensitizes lymphoid tumor cells to olaparib, a poly(ADP-ribose) polymerase-1 inhibitor [28, 29], and ATM is expected to be a biomarker predicting therapeutic response to olaparib in cancer patients. In addition, at present, clinical trials utilizing olaparib in combination with conventional chemotherapy for advanced gastric cancer patients are being initiated, which is concentrated on patients with ATM protein negative. Accordingly, the accumulation of data on ATM expression in gastric cancer is of importance, and our findings concerning ATM could help progress the clinical applications of targeted therapies.

In conclusion, we elucidated the prognostic implications of the expressions of the DDR-related proteins ATM, Chk2, and p53, in gastric carcinoma. We believe that our findings can be used to aid the prediction of clinical outcome and the definition of patient subgroups with an unfavorable prognosis. In addition, our finding that the prognosis was poor even when only 1 of the 3 proteins was aberrantly expressed emphasizes the critical role of DDR-related proteins in the development and progression of gastric cancer.

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