Expression of DNA damage response proteins in gastric cancer: Comprehensive protein profiling and histological analysis

HIROKI ARAI[1,2], RYUICHI WADA[1], KOUSUKE ISHINO[1], MITSUHIRO KUDO[1], EIJI UCHIDA[2] and ZENYA NAITO[1]

Departments of [1]Integrated Diagnostic Pathology and [2]Gastrointestinal Hepato-Biliary-Pancreatic Surgery, Nippon Medical School, Tokyo 113-8602, Japan

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Abstract. Gastric cancer is the third major cause of cancer-related mortality in Japan. The aim of this study was to identify a factor implicated in the biology of gastric cancer by comprehensive protein profiling. Protein profiling was carried out by liquid chromatography-tandem mass spectrometry, using formalin-fixed paraffin-embedded specimens of 17 gastric cancer cases. Pathway analysis and orthogonal partial least square-discriminant analysis suggested the significant expression of ribonucleoproteins, heterogeneous nuclear ribonucleoproteins, interleukin binding factor 2 (ILF2), KU70 and KU80, which are involved in DNA damage response (DDR). Thus, the expression and phosphorylation levels of KU70, ILF2, CHK1 and CHK2 were examined by immunohistochemistry in 42 cases of gastric cancer. The expressions of ILF2 and CHK1 were unaffected in all cases. The expression and phosphorylation of CHK2 were absent in 2 cases. Despite the expression of proteins, the phosphorylation of KU70 and CHK2 appeared to be impaired in 1 and 4 cases, respectively. In 7 out of 42 cases (17%), DDR appeared to be impaired. Recurrence was noted in 2 out of these 7 cases (29%), whereas the recurrence was noted in 2 out of the remaining 35 cases (6%). The expression levels of KU70, ILF2, CHK1, CHK2 and TP53 were further examined in 4 gastric cancer cell lines. The expression and phosphorylation levels following exposure to ultraviolet radiation were abnormal in the 3 cell lines. The normal consecutive phosphorylation of CHK1 and CHK2, the upregulation of TP53 and an increase in apoptotic cell death following exposure to ultraviolet radiation was detected only in one cell line, suggesting that the preserved functions of DDR and TP53 are necessary for the determination of cell fate. It is thus suggested that DDR plays an important role in the pathobiology of gastric cancers.

Introduction

Gastric cancer is the third major cause of the cancer-related mortality in Japan. Owing to endoscopic examination and surgery combined with chemotherapy, the prognosis of patients with gastric cancer has improved (1,2). However, there are still cases of gastric cancer which are diagnosed at an advanced stage, and in these cases, prognosis is unfavorable. To date, the factors that are involved in the pathogenesis and the progression of the gastric cancer have not yet been fully elucidated.

For the elucidation of the pathogenesis of the disease, the comprehensive profiling of proteins, DNA, RNA and metabolites has been carried out (3,4). This profiling would provide invaluable information useful for the identification of molecules of therapy, diagnosis and tumor biology. The comprehensive profiling of proteins and mRNAs has been carried out in gastric cancers (5-9). These molecular studies have revealed that gastric cancers are classified into 4 subtypes: The CpG island methylator phenotype, hypermutated, genomically stable and chromatin instability (6). These subtypes appear to represent the pathogenetic differences of gastric cancers. It is expected that the elucidation of the pathogenesis of gastric cancers leads to the development of specific treatment (8). Molecular information is required for the development of individualized therapy (2).

In the present study, the comprehensive protein profiling of gastric cancers was carried out using protein samples extracted from formalin-fixed paraffin-embedded (FFPE) specimens. Bioinformatics analyses suggested the significance of the expression of proteins in DNA damage response (DDR). DDR

Correspondence to: Dr Ryuichi Wada, Department of Integrated Diagnostic Pathology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan
E-mail: w-ryuichi@nms.ac.jp

Abbreviations: DDR, DNA damage response; ILF2, interleukin binding factor 2; hnRNPs, heterogeneous nuclear ribonucleoproteins; FFPE, formalin-fixed paraffin-embedded; IPA, Ingenuity Pathway Analysis; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NSAF, normalized spectral abundance factor; OPLS-DA, orthogonal partial least squares discriminant analysis; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; UV, ultraviolet; XRCC, X-ray repair cross complementing

Key words: gastric cancer, DNA damage response, KU70, CHK1, CHK2, TP53, apoptosis

is the consecutive sequence from the detection of damaged DNA, the aggregation of transducers and modulators at the damaged site, cell cycle arrest and the repair of the damaged DNA. The rapid and uncontrolled progression of the cell cycle of carcinoma cells causes frequent errors in genome duplication, and the defect in DDR may enhance genetic instability and lead to the progression of carcinoma (10). In this study, the expression of molecules of DDR and the phosphorylation state of proteins were examined in cases of gastric cancer and in cultured gastric cancer cell lines. The preserved expression and phosphorylation of DDR proteins appeared to be associated with a favorable prognosis.

Materials and methods

Cases of human gastric cancer. Cases of gastric cancer were retrieved from the archives of the pathology records of Nippon Medical School Hospital (Tokyo, Japan) from 2011 to 2016. In total, 17 cases were used for the profiling of expressed proteins, and they were selected randomly from the pathology records. A total of 42 cases were used for the histological and immunohistochemical analyses. The cases were randomly selected from the cases of gastric cancer at pathological stages from I to III. The patients underwent gastrectomy, but they did not receive chemotherapy or radiation therapy prior to surgery. The study was conducted according to the declaration of Helsinki and the Japanese Society of Pathology. This study was approved by the Ethics Committee of Nippon Medical School Hospital (#29-06-764). Informed consent was obtained from all patients.

Comprehensive profiling of protein expression. FFPE specimens from 17 cases of gastric cancer were used for the comprehensive profiling of proteins by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The sections of gastric cancer (10-μm-thick) were deparaffinized in xylene and rehydrated through a graded alcohol series. Following staining with hematoxylin, the cancer tissues were dissected out under a stereoscopic microscope (AZ-STDM; Nikon Co., Tokyo, Japan) and lysed in buffer with 100 mM Tris-HCl (pH 9.0)/12 mM deoxycholate/12 mM lauroyl sarcosine. Following the quantification of the protein concentration by Bradford method using Bio-Rad Protein assay (Bio-Rad, Laboratories, Inc., Hercules, CA, USA), 10 μg of extracted protein was reduced in 11.4 mM dithiothreitol (DTT)/1.8 mM Tris (2-carboxyethyl) phosphine hydrochloride and alkylated in 54 mM iodoacetamide. It was further digested with proteomics-grade trypsin (Agilent Technologies Inc., Santa Clara, CA, USA) at 37˚C for 24 h, and the protein was purified with a PepClean C-18 spin column (Thermo Fisher Scientific, K.K., Tokyo, Japan). In total, 2 μg of digested protein was injected into a Nano cHiPLC Trap column (0.2x0.5 mm, ChromXP C18-CL 3 μm) and further separated through a Eksigent nano LC Ekspert415 system using a reverse-phase C-18 column (0.075x150 mm, ChromXP C18-CL 3 μm) (all from K.K. Sciex Japan, Tokyo, Japan). The protein solution was run with the gradient concentration of acetonitrile from 2 to 32% in 0.1% formic acid in acetonitrile at a flow rate of 300 nl/min for 120 min. Eluted peptides were analyzed by Triple TOF 5600$^+$ mass spectrometer (K.K. Sciex Japan). The data of 10 most intense peaks of each full MS scan were acquired. All MS/MS spectral data were analyzed by MASCOT 2.4 (Matrix Science K.K., Tokyo, Japan) with SwissProt 2015_02. The following parameter settings were used: Trypsin cleavage; two missed cleavage sites allowed for cysteine carbamidomethylation (C-terminus) and methionine oxidation (N-terminus). Peptide mass tolerance was set to ±50 ppm, and fragment MS/MS tolerance were set to 0.05 Da. The amounts of identified proteins were expressed as normalized spectral abundance factor (NSAF).

Bioinformatics analyses. Protein expression was analyzed by hierarchical clustering, orthogonal partial least squares-discriminant analysis (OPLS-DA) and Ingenuity Pathway Analysis (IPA). Analyses were done using NSAF of protein.

Hierarchical clustering was carried out using Cluster 3.0 and visualized using TreeView software (Howard Hughes Medical Institutes, University of California at Berkeley, Berkeley, CA, USA). Analysis with OPLS-DA was carried out using SIMCA version 14 (Umetrics, Umea, Sweden) to identify proteins which have a significant influence on the magnitude {p[1]} and reliability {p(corr)[1]} to separate into 2 clusters. The results were visualized as S-PLOT, a scatter plot with magnitude {p[1]} as the x-axis and reliability {p(corr)[1]} as the y-axis. The reliability, which was ≥0.6 and ≤-0.6 was considered significant. Pathways of expressed proteins were analyzed by IPA (Qiagen, Redwood City, CA, USA). The scores of networks were calculated by the numbers of focus proteins. The percentages of the number of reliable proteins evaluated by OPLS-DA in the number of focus proteins of each network identified by IPA were then calculated. It was expected that the combined analysis of OPLS-DA and IPA was useful to identify the biologically significant proteins from the profiled proteins.

Histological examination of human gastric cancers. A total of 42 cases of gastric cancer were used for the histological and immunohistochemical analyses. The histological subtypes were classified according to the classification by Lauren (11). Pathological T-factors, lymphovascular invasion and lymph node metastasis were also reviewed. The review of the histology was performed by 3 investigators (H.A., R.W. and Z.N.).

Immunohistochemistry. Immunohistochemistry was performed using the polymer-based two-step method. Briefly, the paraffin-embedded sections were deparaffinized and hydrated in phosphate-buffered saline (PBS). After the blocking of endogenous peroxidase, the sections were pretreated, if necessary, and then incubated with primary antibodies listed in Table I at 4˚C overnight. The sections were then incubated with Simple Stain MAX-PO (R) for rabbit primary antibody or (M) for mouse primary antibody (Nichirei Inc., Tokyo, Japan). Peroxidase activity was visualized by diaminobenzidine.

Culture of gastric cancer cell lines and exposure to ultraviolet (UV) radiation. Four gastric cancer cell lines, NS-8, MKN-7, NUGC-4 and KATO-III, were used in this study. The cells were cultured in DMEM with 10% fetal bovine serum and penicillin/streptomycin. The cells were exposed to UV radiation at a dose of 40 J/m^2. At 2 h following exposure, the cells

Table I. The antibodies used in the present study.

Antibody	Clone	Species	Dilution WB	IHC	PT	Company/provider
KU70	EPR4026	Rabbit	1:10,000	1:2,000	-	Abcam K.K., Tokyo, Japan
p-KU70 (Ser5)	ab61783	Rabbit	1:10,000	1:2,000	TB	Abcam K.K., Tokyo, Japan
ILF2	ab28772	Rabbit	1:1,000	-	CB	Abcam K.K., Tokyo, Japan
CHK1	2G1D5	Mouse	1:1,000	1:500	TB	Cell Signaling Technology, Japan, K.K. Tokyo, Japan
p-CHK1 (Ser317)	D92H3	Rabbit	1:1,000	1:5,000	CB	Cell Signaling Technology, Japan, K.K. Tokyo, Japan
CHK2	D9C6	Rabbit	1:1,000	1:1,000	CB	Cell Signaling Technology, Japan, K.K. Tokyo, Japan
p-CHK2 (Thr68)	C13C1	Rabbit	1:1,000	1:1,000	TB	Cell Signaling Technology, Japan, K.K. Tokyo, Japan
TP53	DO7	Mouse	1:1,000	-	CB	Agilent Technologies Japan, Ltd. Tokyo, Japan

CB, citrate buffer (pH 6.0) at 121˚C for 15 min; IHC, immunohistochemistry; PT, pretreatment; TB, Tris buffer (pH 9.0) at 121˚C for 15 min; WB, western blot analysis. ILF2, interleukin binding factor 2.

were washed with PBS and lysed in 50 mM Tris-HCl (pH 7.6). The sample was then sonicated for 10 min.

Western blot analysis. The cell lysate was electrophoresed and blotted onto a polyvinylidene difluoride membrane. After the blocking in 5% skim milk in buffer of 0.2 M Tris-HCl (pH 7.6)/150 mM NaCl/0.01% Tween-20, the membrane was incubated with the primary antibodies listed in Table I overnight. The membrane was then incubated with peroxidase-labeled anti-mouse immunoglobulin antibody (#A106PU) or anti-rabbit immunoglobulin antibody (#A102PU) (both from American Qualex Scientific Products, San Clemente, CA, USA), and the peroxidase activity was detected as chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, K.K.).

Analysis of apoptotic cell death in cultured cells. Cell death was examined using the Apoptosis, Necrosis and Healthy Cell Quantitation kit (Biotium, Inc., Hayward, CA, USA). Briefly, the cultured cells were washed with PBS, and incubated with a mixture of FITC-labeled Annexin V and Hoechst 33342 at room temperature for 15 min. Apoptosis was identified when cells were stained positive for FITC. The frequency of apoptotic cell death was calculated by dividing the number of apoptotic cells by the total number of cells. A total of at least total 500 cells was counted.

Statistical analysis. All quantitative data are expressed as the means ± SD. The distribution of cases was analyzed by the Chi-square test with Fisher's correction. The data of 2 groups were analyzed by the Mann-Whitney U test. P-values <0.05 was considered to indicate statistically significant differences. All statistical analyses were carried out using JMP 13.0 software (SAS Institute Japan, Ltd., Tokyo, Japan).

Results

Comprehensive profiling of protein expression and bioinformatics analyses. The comprehensive profiling of proteins was performed with the proteins purified from the FFPE samples of 17 cases of gastric cancer. A total of 5,338 proteins were identified by LC-MS/MS. NSAFs of housekeeping proteins, such as β-actin and histones were comparable among the cases (data not shown).

Among the 5,338 proteins, 483 proteins (9%) were expressed in all 17 cases. The other proteins were not detected at least one case. Unsupervised hierarchical clustering was performed with these 483 proteins (Fig. 1A). The gastric cancers were separated into 2 major clusters. The left cluster included 9 cases, and the right cluster included 8 cases. There was no significant difference as regards age, location, pathological T factor and pathological stage between the 2 clusters (Table II). All cases in the right cluster were male. The intestinal subtype was predominant in the left cluster, whereas the diffuse subtype was predominant in the right cluster.

To identify the proteins reliable to separate the left and right clusters, OPLS-DA was carried out with NSAF of 483 proteins and visualized by S-PLOT (Fig. 1B). The reliability {p(corr)[1]} of 33 proteins was ≥0.6, and it was considered that they had significant reliability to discriminate the right cluster (Table III). On the other hand, the reliability of 46 proteins was ≤-0.6, and they had significant reliability for the discrimination of the left cluster (Table IV).

Networks of proteins were analyzed by IPA with NSAF of 483 proteins, and a total of 24 networks was identified. To identify proteins which are biologically significant in gastric cancer, protein expression was further analyzed by combining the results of OPLS-DA and IPA. The proteins identified as reliable for the discrimination of right clusters were enriched in

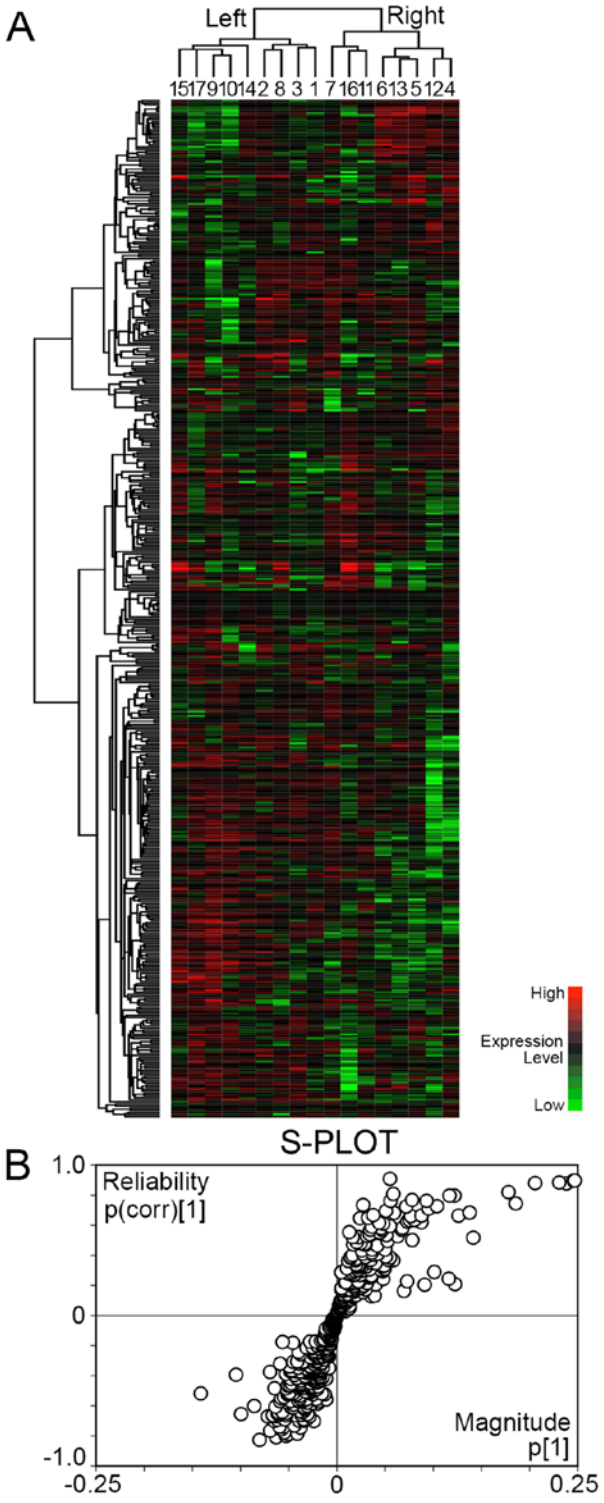

Figure 1. Comprehensive analysis of expressed proteins. (A) Hierarchical cluster analysis of protein expression in gastric cancer. The cases of gastric cancers were separated into 2 major clusters. (B) S-PLOT of magnitude (p[1]) and reliability {p(corr)[1]} of proteins analyzed by orthogonal partial least squares-discriminant analysis.

Table II. Clinicopathological characteristics of the patients with gastric cancer in the left and right clusters.

	Left cluster (n=9)	Right cluster (n=8)	P-value
Age (years)	72±12	74±7	ns
Sex			
Male	4	8	P<0.05
Female	5	0	
Location			
Upper	4	1	ns
Middle	1	1	
ML	1	2	
Lower	3	4	
Lauren classification			
Intestinal	6	2	P<0.05
Mixed	3	1	
Diffuse	0	5	
pT			
pT1	6	5	ns
pT2	1	3	
pT3	2	0	
pT4	0	0	
Stage			
I	6	5	ns
II	2	3	
III	1	0	
IV	0	0	

ML, middle to lower; ns, not significant.

networks 2 and 19 (Table V). The percentages of proteins identified by OPLS-DA (double underlined proteins in Table V) in the focus molecules of network 2 and 19 were 23% (7/31) and 54% (7/13), respectively. They were intermediate filaments, matrix proteins and their binding proteins. Some were associated with cell death. The combined networks of 2 and 19 are shown in Fig. 2A. Among the 322 connections, 53 connections (pink-colored lines in Fig. 2A, 17%) were interactions between the proteins in 2 networks. On the other hand, the proteins identified as reliable for the discrimination of the left cluster by OPLS-DA were enriched in networks 1, 3, 5 and 9 (Table V). The percentages of proteins identified by OPLS-DA (underlined proteins in Table V) in the focus molecules of networks 1, 3, 5 and 9 were 27% (9/33), 29% (8/28), 15% (4/27) and 16% (4/25), respectively. Combined networks formed tight connections (Fig. 2B), and 231 pink-colored connections out of 557 connections (42%) were interactions among 4 networks. The focus molecules were tightly linked, suggesting the close functional associations among the proteins (Fig. 2B). The majority of these proteins were ribosomal proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). The networks also included XRCC5 (KU80), XRCC6 (KU70) and interleukin enhancer binding protein 2 (ILF2).

KU70 and KU80 are sensors of damaged DNA (12). ILF2 regulates the splicing of mRNAs, and it has been shown that ILF2 regulates RNA processing of genes involved in DDR (13). hnRNPs and ribosomal proteins stabilize mRNAs and regulate the splicing and translation of mRNAs, and it was recently shown that hnRNPs and ribosomal proteins stabilize the genome when DNA is damaged (14). The tight connections

Table III. Proteins identified by orthogonal partial least squares-discriminant analysis (the reliability is ≥0.6).

Protein	Magnitude p[1]	Reliability p(corr)[1]
INA	0.055052	0.909151
ACTA2	0.246902	0.897744
ACTC1	0.245703	0.896767
ACTA1	0.230718	0.884632
VIM	0.205729	0.880668
ACTB	0.238275	0.878102
ACTBL2	0.178069	0.820505
PRPH	0.058522	0.808254
TPM1	0.116885	0.797396
TPM2	0.121969	0.797050
FLNA	0.078055	0.768442
TPM3	0.093025	0.762556
CSRP1	0.055634	0.761816
TAGLN	0.185655	0.745214
FLNC	0.027218	0.735016
FTL	0.103966	0.726628
ANXA6	0.071863	0.724106
ANXA5	0.095132	0.710682
MSN	0.050898	0.695238
DES	0.137757	0.684517
S100A4	0.059389	0.682374
MYH10	0.037659	0.667677
MYL6	0.087813	0.664537
POTEKP	0.126213	0.664068
POTEE	0.076739	0.660707
RDX	0.032470	0.649216
PKLR	0.024108	0.646927
COL6A3	0.080483	0.634928
COL6A2	0.075606	0.632119
COL6A1	0.072951	0.624386
LUM	0.087745	0.619709
POTEI	0.043763	0.616854
VCL	0.048017	0.610764

Table IV. Proteins identified by orthogonal partial least squares-discriminant analysis (the reliability is ≤-0.6).

Protein	Magnitude p[1]	Reliability p(corr)[1]
HYOU1	-0.031015	-0.600712
NME1	-0.085839	-0.601319
RPS16	-0.063733	-0.606282
ATIC	-0.033951	-0.609055
HNRNPA1L2	-0.045016	-0.610854
ETFB	-0.047257	-0.611190
PDLIM1	-0.034208	-0.612325
RNPEP	-0.032576	-0.612375
CUTA	-0.032975	-0.624701
RPS5	-0.038159	-0.635456
RPSA	-0.056624	-0.640193
RPS13	-0.061149	-0.641738
XRCC5	-0.035668	-0.643890
HNRNPC	-0.052836	-0.649307
RPS19	-0.061370	-0.653405
TXN	-0.099468	-0.654578
SFPQ	-0.030426	-0.656324
RPL4	-0.036438	-0.660385
HNRNPU	-0.036768	-0.661809
RPS18	-0.066594	-0.662418
PSMA6	-0.052565	-0.663324
EEF1B2	-0.043041	-0.663354
PHB	-0.065979	-0.667600
C1QBP	-0.062352	-0.669720
DDX3X	-0.028476	-0.670196
HSPD1	-0.071720	-0.670892
PABPC1	-0.034400	-0.690058
HSD17B10	-0.054045	-0.691120
NANS	-0.031787	-0.693264
PDCD6	-0.061551	-0.693424
HNRNPM	-0.034036	-0.699508
SYNCRIP	-0.039143	-0.699748
AKR1A1	-0.064921	-0.700549
CTNND1	-0.023981	-0.701687
HNRNPH1	-0.044206	-0.731575
XRCC6	-0.059573	-0.739444
RPS4X	-0.055168	-0.739747
RPS23	-0.037777	-0.745383
CCT2	-0.057704	-0.755868
ETHE1	-0.069280	-0.765860
ILF2	-0.054563	-0.777686
CIRBP	-0.041115	-0.785409
HNRNPF	-0.050593	-0.787704
EEF2	-0.052731	-0.800286
VCP	-0.062993	-0.809144
RPS7	-0.080280	-0.826483

of these proteins raise the possibility of the involvement of DDR in gastric cancer. DDR is exerted by the orchestration of molecules, sensors of damaged DNA, transducers, mediators and effectors to repair the damaged DNA and to determine the fate of the cell (Fig. 3). The transduction pathway is exerted by the phosphorylation of proteins. Thus, in this study, the expression and phosphorylation of key proteins of DDR were examined by immunostaining of 42 cases of gastric cancer.

Clinicopathological characteristics and DNA damage response proteins in gastric cancers. The clinicopathological characteristics of 42 cases of gastric cancer are summarized in Table VI. The age of the patients varied from 53 to 86 years. In total, 35 cases were male, and 7 cases were female. Histological subtypes were evaluated by Lauren classification, and the number of cases of intestinal, diffuse and mixed types were 20 (48%), 12 (29%) and 10 (23%) cases, respectively (Table VI).

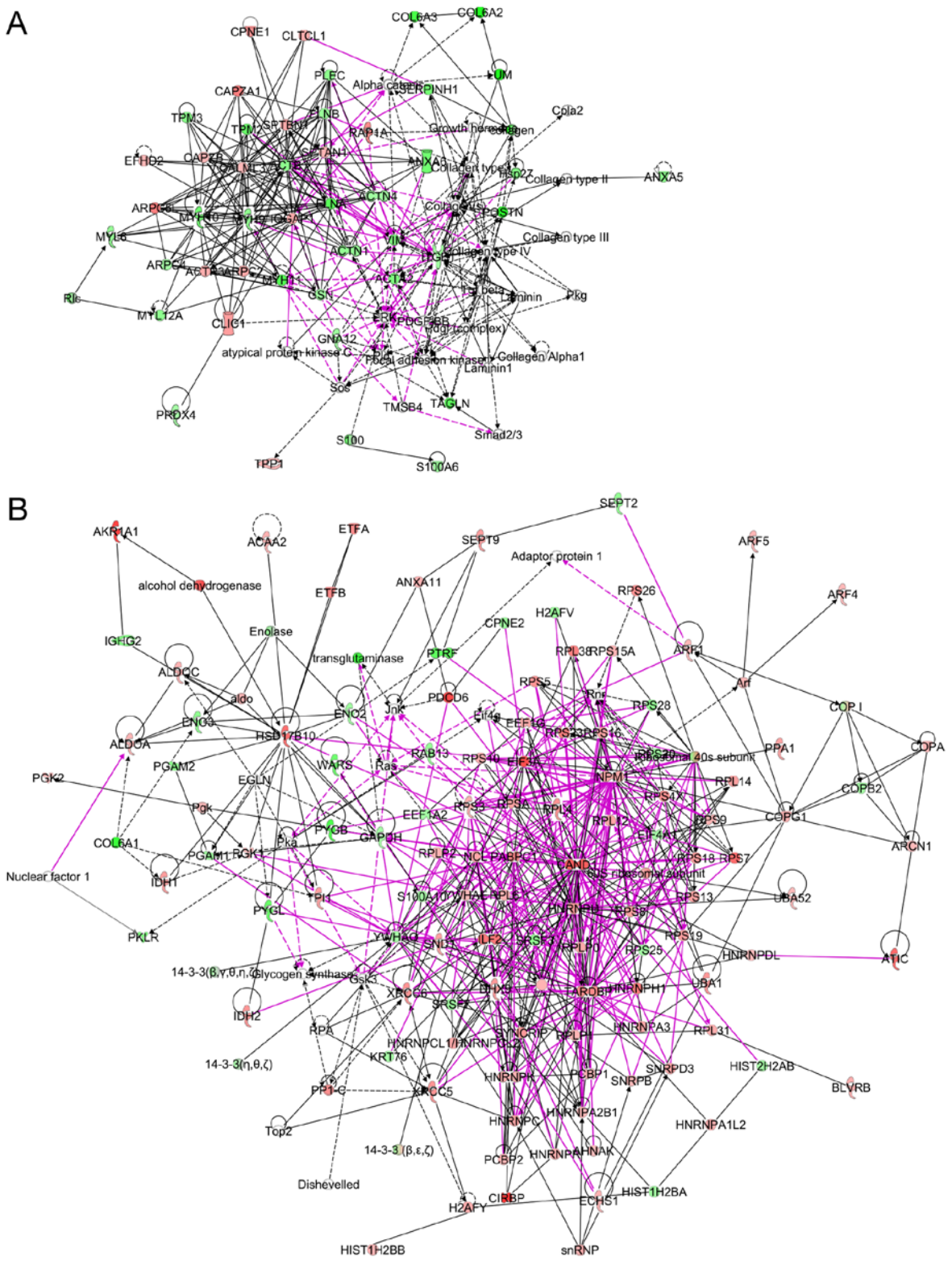

Figure 2. Ingenuity Pathway Analysis of the proteins in gastric cancer. (A) Combined networks of 2 and 19. These networks were enriched with the proteins reliable to separate the right cluster. In total 53 (pink-colored) out of a total of 322 connections (17%) represent the interactions between the focus molecules in 2 networks. (B) Combined networks of 1, 3, 5 and 9. They were enriched with the proteins reliable to separate the left cluster. Out of the total 557 connections, 231 connections (pink-colored, 42%) represent the interactions among the focus molecules in 4 networks.

Immunohistochemistry was performed using antibodies against KU70, pKU70, CHK1, pCHK1, CHK2 and pCHK2 (Fig. 4). The expression of KU70 was found in all cases, however, the phosphorylation was not apparent in one case (case 34) (Fig. 4 and Table VI). The expression of CHK1 and protein phosphorylation was observed in all cases. In 4 cases (cases 24, 25, 39 and 41) (Fig. 4 and Table VI), the expression of CHK2 was found in tumor cells; however, the positive reaction of pCHK2 was not apparent in tumor cells. The expression of CHK2 was not found in 2 cases (cases 23

Table V. Networks identified by Ingenuity Pathway Analysis.

ID	Molecules in network	Score	Focus molecules	Top diseases and functions
1	60S ribosomal subunit, **CAND1**, **CIRBP**, **EEF1A2**, **EEF1G**, **EIF3A**, **HNRNPA1L2**, **HNRNPCL1/HNRNPCL2**, **HNRNPDL**, **HNRNPK**, **HNRNPR**, **HNRNPU**, **KRT76**, **PABPC1**, **PCBP1**, **PCBP2**, Ras, **RPL4**, **RPL14**, **RPL38**, **RPLP0**, **RPS3**, **RPS8**, **RPS9**, **RPS10**, **RPS16**, **RPS19**, **RPS25**, **RPSA**, **SND1**, **SRSF3**, **SRSF7**, **SYNCRIP**, **TARDBP**, **UBA52**	57	33	RNA post-transcriptional modification, protein synthesis, Cancer
2	**ACTB**, **ACTN1**, **ACTN4**, **ACTR3**, **ANXA6**, **ARPC2**, **ARPC4**, **ARPC5L**, **CALML3**, **CAPZA1**, **CAPZB**, **CLIC1**, **CLTCL1**, **CPNE1**, **EFHD2**, ERK, **FLNA**, **FLNB**, **GSN**, **IQGAP1**, **MYH9**, **MYH10**, **MYH11**, **MYL6**, **MYL12A**, **PLEC**, **PRDX4**, Rlc, S100, **S100A6**, **SPTAN1**, **SPTBN1**, TMSB4, **TPM2**, **TPM3**	52	31	Cellular assembly and organization, xellular function and maintenance, cell-to-cell signaling and interaction
3	**ARCN1**, Arf, **ARF1**, **ARF4**, **ARF5**, **ATIC**, COPI, **COPA**, **COPB2**, **COPG1**, **ECHS1**, **EIF4A1**, Eif4g, **H2AFV**, **HNRNPA3**, **HNRNPH1**, **NPM1**, nuclear factor 1, Pka, **PKLR**, **PPA1**, **RAB13**, ribosomal 40s subunit, Rnr, **RPS5**, **RPS7**, **RPS13**, **RPS18**, **RPS20**, **RPS23**, **RPS26**, **RPS28**, **RPS15A**, **RPS4X**, **UBA1**	44	28	Cancer, cell death and survival, organismal injury and abnormalities
5	**ACAA2**, adaptor protein 1, **AKR1A1**, alcohol dehydrogenase, aldo, **ALDOA**, **ALDOC**, **ANXA11**, **COL6A1**, **CPNE2**, EGLN, **ENO2**, **ENO3**, enolase, **ETFA**, **ETFB**, **GAPDH**, **HSD17B10**, **IDH1**, **IDH2**, **IGHG2**, Jnk, **PDCD6**, **PGAM1**, **PGAM2**, Pgk, **PGK1**, **PGK2**, **PTRF**, **PYGB**, **PYGL**, **SEPT2**, **SEPT9**, **TPI1**, transglutaminase	42	27	Cancer, cell death and survival, organismal injury and abnormalities
9	14-3-3 (β,ε,ζ), 14-3-3 (β,γ,θ,η,ζ), 14-3-3 (η,θ,ζ), **AHNAK**, **H2AFY**, **BLVRB**, **DHX9**, dishevelled, glycogen synthase, Gsk3, **HIST1H2BA**, **HIST1H2BB**, **HIST2H2AB**, **HNRNPA2B1**, **HNRNPC**, **ILF2**, **NCL**, PP1-C, RPA, **RPL6**, **RPL12**, **RPL31**, **RPLP1**, **RPLP2**, **S100A10**, snRNP, **SNRPB**, **SNRPD3**, **SRSF1**, Top2, **WARS**, **XRCC5**, **XRCC6**, **YWHAE**, YWHAQ	37	25	RNA post-transcriptional modification, cell morphology, cellular function and maintenance
19	**ACTA2**, α catenin, **ANXA5**, atypical protein kinase C, **COL6A2**, **COL6A3**, collagen, collagen α1, collagen type I, collagen type II, collagen type III, collagen type IV, collagen(s), Cpla2, focal adhesion kinase, **GNA12**, growth hormone, Hsp27, **ITGB1**, laminin, laminin1, **LUM**, Pdgf, PDGF BB, Pkg, Pld, **POSTN**, **RAP1A**, **SERPINH1**, Smad2/3, Sos, **TAGLN**, Tgfβ, **TPP1**, **VIM**	14	13	Developmental disorder, organismal injury and abnormalities, connective tissue disorders

The proteins shown in bold are focus molecules. The underlined text indicates that the reliability {p(corr)[1]} evaluated by the orthogonal partial least square-discriminant analysis is <-0.6, and the double underlined text indicates that the reliability is >0.6.

and 33) (Fig. 4 and Table VI), and pCHK2 was also absent. The expression and phosphorylation of proteins of the DDR pathway appeared to be impaired in 7 (cases 23, 24, 25, 33, 34, 39 and 41) out of the 42 cases (17%) (Table VI).

Recurrence was noted in 2 out of 7 cases (29%), in which expression and phosphorylation were impaired (cases 23 and 39) (Table VI). One patient succumbed to the disease. On the other hand, out of remaining 35 cases, in which the DDR pathway was intact, recurrence was noted in 2 cases (6%, cases 5 and 7) (Table VI).

DNA damage response proteins and apoptotic cell death in gastric cancer cell lines. KU70 was expressed in all the cell lines apart from the NS-8 cells (Fig. 5). The differences in the expression levels of KU70 were not evident among the other cells. The expression of ILF2 was observed in all cell lines; however, the expression appeared to be decreased in the NS-8 cells. The expression level of CHK1 appeared similar among the cultured cells. CHK2 was overexpressed in the NS-8 cell; however, its molecular size differed from that observed in the other cell lines. The expression of TP53 was not detected in the NS-8 and KATO-III cells. TP53 was overexpressed in the MKN-7 cells, in which the TP53 gene is mutated (15).

At 2 h following exposure to UV radiation, the expression level of KU70 appeared to be unaltered, and its phosphorylation was not evident after 2 h (Fig. 5). The phosphorylation of CHK1 was increased following exposure to UV radiation in cell lines; however, this increase was not evident in the NS-8 cells.

Table VI. Clinicopathological characteristics and immunohistochemical results of the cases of gastric cancer.

No	Age/sex	Lauren	pT	pN	Stage	KU70	pKU70	CHK1	pCHK1	CHK2	pCHK2	Rec	Prognosis
1	53/M	Intestinal	1a	0	IA	+	+	+	+	+	+		
2	55/M	Intestinal	3	1	IIB	+	+	+	+	+	+		
3	56/M	Intestinal	1b1	0	IIA	+	+	+	+	+	+		
4	59/M	Diffuse	4a	0	IIB	+	+	+	+	+	+		
5	62/M	Intestinal	4a	3b	IIIB	+	+	+	+	+	+	+, 3 mo	DOD, 18 mo
6	62/M	Intestinal	4a	2	IIIB	+	+	+	+	+	+		
7	63/M	Intestinal	3	2	IIIA	+	+	+	+	+	+	+, 10 mo	DOD, 33 mo
8	64/M	Diffuse	3	3b	IIIC	+	+	+	+	+	+		
9	65/M	Mixed	4a	1	IIIA	+	+	+	+	+	+		
10	65/M	Intestinal	1b2	1	IB	+	+	+	+	+	+		
11	65/M	Intestinal	1a	0	IA	+	+	+	+	+	+		
12	66/M	Diffuse	2	0	IB	+	+	+	+	+	+		
13	68/M	Mixed	1b2	0	IA	+	+	+	+	+	+		
14	68/M	Intestinal	1a	0	IA	+	+	+	+	+	+		
15	70/M	Mixed	2	3a	IIIA	+	+	+	+	+	+		
16	70/M	Mixed	4a	3a	IIIC	+	+	+	+	+	+		
17	70/M	Intestinal	3	1	IIB	+	+	+	+	+	+		
18	70/M	Mixed	4a	0	IIB	+	+	+	+	+	+		
19	70/M	Intestinal	2	1	IIA	+	+	+	+	+	+		
20	70/M	Intestinal	2	0	IB	+	+	+	+	+	+		
21	70/F	Intestinal	1a	0	IA	+	+	+	+	+	+		
22	71/M	Intestinal	1b1	0	IA	+	+	+	+	+	+		
23	71/M	Diffuse	4a	3b	IIIC	+	+	+	+	-	-	+, 4 mo	DOD, 5 mo
24	73/F	Mixed	3	0	IIA	+	+	+	+	+	-		
25	74/M	Mixed	3	2	IIB	+	+	+	+	+	-		
26	74/M	Intestinal	2	0	IB	+	+	+	+	+	+		
27	75/M	Diffuse	2	1	IIB	+	+	+	+	+	+		
28	75/F	Diffuse	3	3a	IIIB	+	+	+	+	+	+		
29	76/M	Intestinal	1b1	0	IA	+	+	+	+	+	+		
30	76/M	Diffuse	1b2	0	IA	+	+	+	+	+	+		
31	76/M	Diffuse	2	0	IB	+	+	+	+	+	+		
32	76/F	Intestinal	2	0	IB	+	+	+	+	+	+		
33	77/M	Mixed	4a	3a	IIIC	+	+	+	+	-	-		DOOD
34	78/M	Diffuse	1a	0	IA	+	-	+	+	+	+		
35	81/M	Intestinal	1b2	0	IA	+	+	+	+	+	+		DOOD
36	81/M	Diffuse	3	1	IIB	+	+	+	+	+	+		
37	81/M	Diffuse	1b2	2	IIA	+	+	+	+	+	+		
38	82/F	Mixed	1b2	0	IA	+	+	+	+	+	+		
39	83/F	Mixed	3	2	IIIB	+	+	+	+	+	-	+, 6 mo	DOD, 12 mo
40	85/M	Intestinal	1b1	0	IA	+	+	+	+	+	+		
41	86/M	Diffuse	2	1	IIA	+	+	+	+	+	-		
42	86/F	Intestinal	1a	0	IA	+	+	+	+	+	+		

DOD, died of disease; DOOD, died of other disease; F, female; M, male; mo, month; Rec, recurrence.

The expression level of CHK2 remained unaltered following exposure to UV radiation, and CHK2 was phosphorylated in the cultured cells apart from the NS-8 cells. Overexpressed TP53 in the MKN-7 remained unaltered following exposure to UV radiation. In the NUGC-4 cells, the expression of TP53 was upregulated at 2 h following exposure to UV radiation.

Morphologically, some cultured cells appeared to be swollen at 2 h following exposure to UV radiation (Fig. 6A). The amount of apoptotic cells was increased following exposure to UV radiation in all cell lines apart from the KATO-III cells. The increase in apoptotic cell death following exposure to UV radiation appeared to be pronounced in the NUGC-4

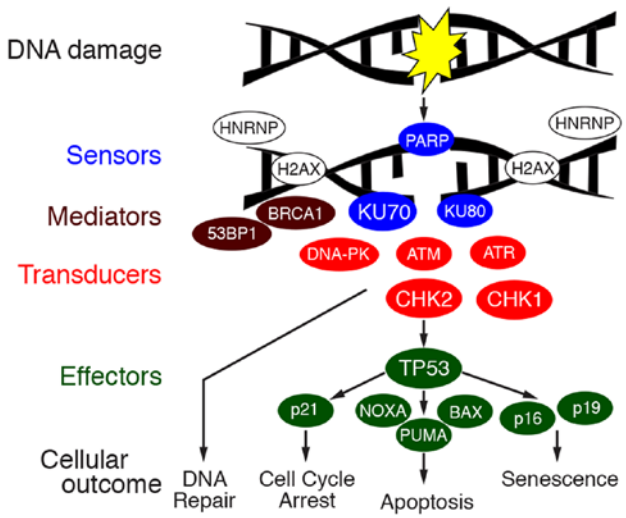

Figure 3. Overview of pathway of DNA damage response. The pathway consists of sensors of damaged DNA, transducers, mediators and effectors to decide the cell fate. Heterogeneous nuclear ribonucleoproteins stabilize the damaged DNA. The schema is modified from previous studies (12,14).

cells, although the difference did not reach statistical significance (Fig. 6B).

Discussion

The present study revealed the biological and clinical significance of DDR in gastric cancer. The comprehensive protein profiling and bioinformatics analyses suggested the possible involvement of DDR in gastric cancer. The expression and phosphorylation of the proteins of DDR were verified and examined in 42 cases of gastric cancer. Among these cases, the expression and phosphorylation of proteins of DDR appeared abnormal in 7 out of 42 cases (17%). Although the number of cases was limited, the recurrence in the cases with an impaired expression and the phosphorylation of DDR proteins was more frequent than that in cases with a preserved expression and phosphorylation of the proteins. In the cultured gastric cancer cells, the effective induction of apoptotic cell death following exposure to UV radiation was observed in one cell line with a preserved expression and phosphorylation of DDR proteins.

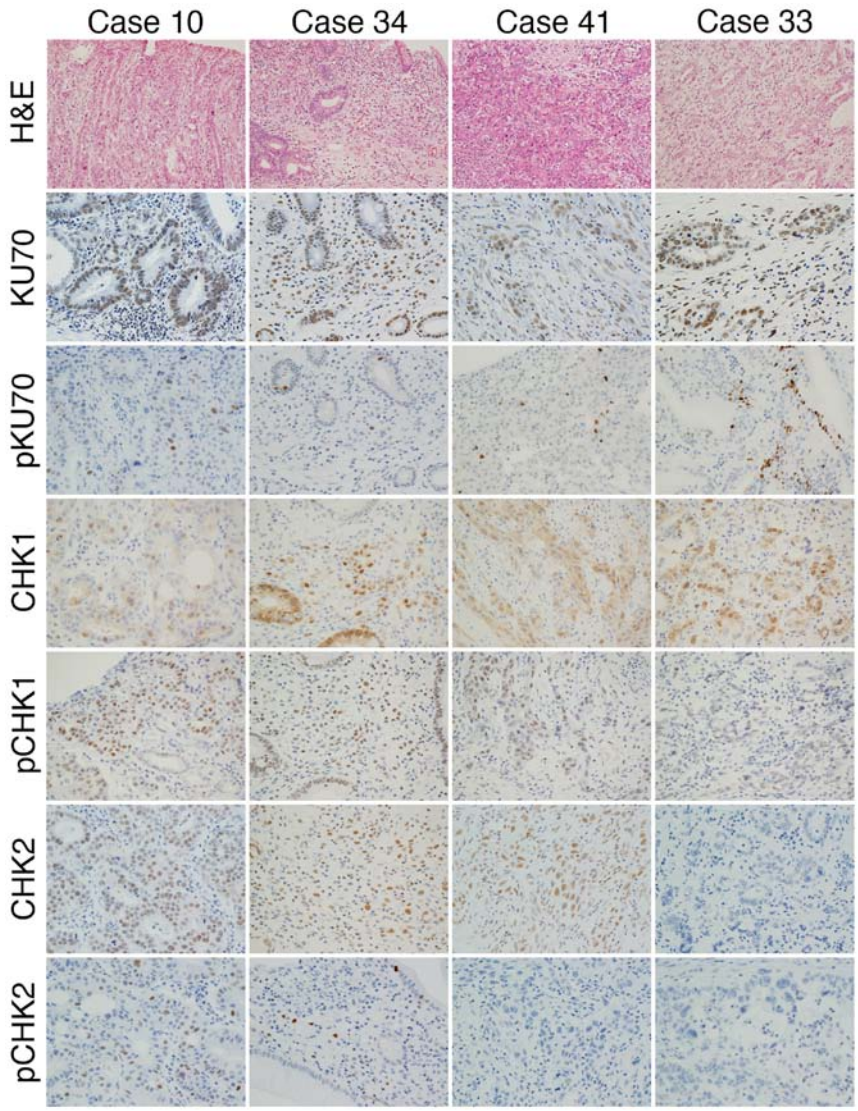

Figure 4. Immunohistochemistry of proteins involved in the DNA damage response pathway in 42 cases of gastric cancers. The representative positive immunostaining images of gastric cancer are shown in the left panels (case 10). The representative immunostaining results of cases, which was negative for pKU70 (case 34), for pCHK2 (case 41), and for both CHK2 and pCHK2 (case 33).

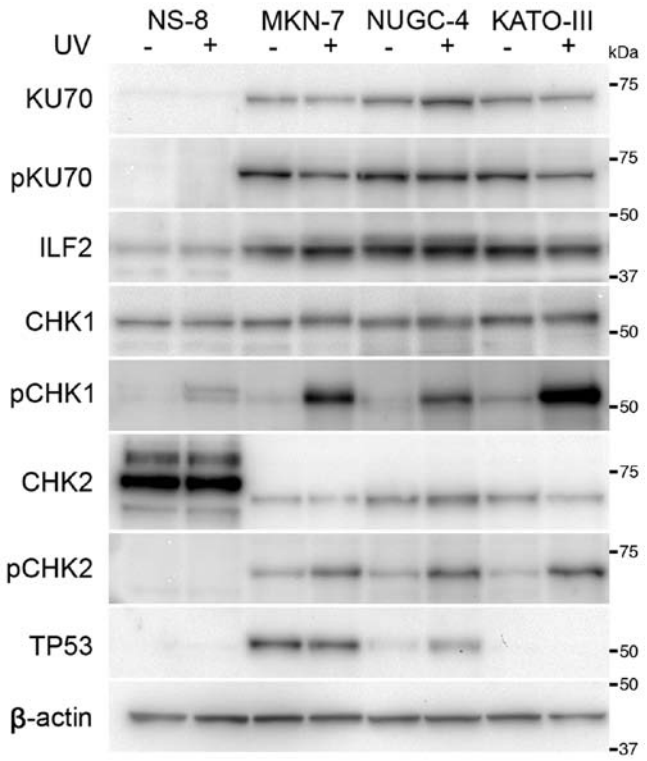

Figure 5. The expression and phosphorylation of DNA damage response proteins in cultured gastric cancer cell lines. The proteins were extracted from cells not exposed to ultraviolet radiation (-) or those exposed to ultraviolet radiation (+) at 2 h after the exposure. The expression of KU70 was absent, and that of interleukin binding factor 2 (ILF2) was decreased in the NS-8 cells. The aberrant overexpression of CHK2 was also noted in the NS-8 cells. The aberrant expression TP53 was observed in the NS-8, MKN-7 and KATO-III cells.

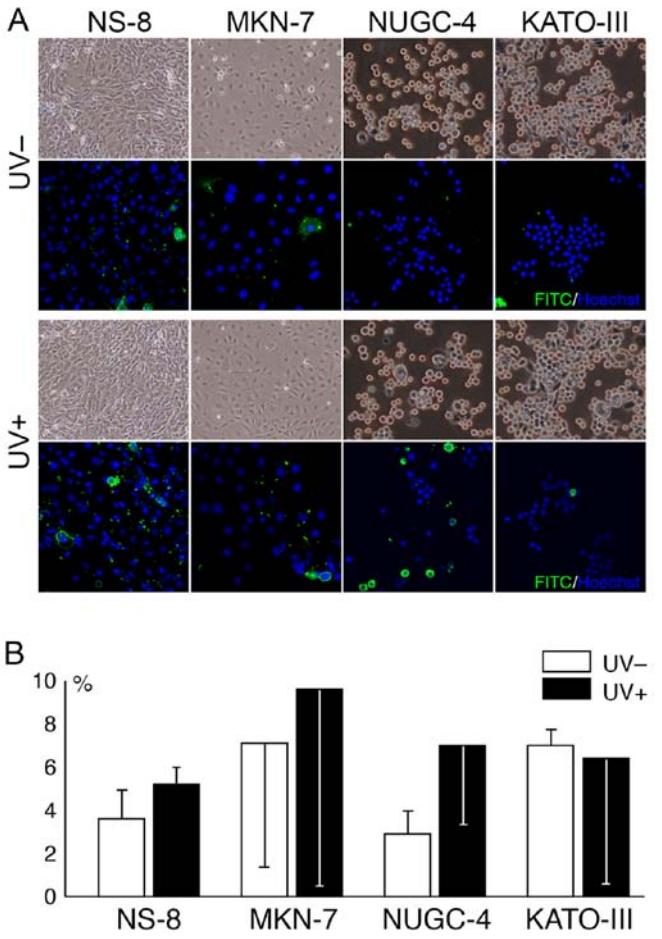

Figure 6. Morphological changes and apoptotic cell death in gastric cancer cell lines not exposed to ultraviolet (UV-) and those exposed to ultraviolet (UV+) at 2 h after the exposure. (A) Some cells appeared to be swollen following exposure to ultraviolet radiation. Apoptotic cells were identified by membranous staining with Annexin V (green color). The cells were counterstained with Hoechst 33342 (blue). (B) Frequency of apoptotic cell death in cultured cell lines. The data represent the means ± SD. Apoptotic cell death following exposure to ultraviolet radiation was increased in the NUGC-4 cells.

The comprehensive profiling of molecules in gastric cancers has been reported in the literature (5,6,16). It was indicated that gastric cancers are categorized into 4 subtypes, CpG island methylator phenotype, hypermutated, genomically stable and chromatin instability (6). However, definitive molecular alterations, which are involved in pathogenesis and progression of gastric cancer, have not been identified. In the present study, for the identification of molecules or pathway commonly involved in the pathobiology of gastric cancer, the proteins that were expressed in all 17 cases gastric cancer were analyzed. The number of these proteins constituted 9% (483/5,338) of the total proteins identified by LC-MS/MS, and the proteins with a subtle expression, as well as proteins expressed specifically in certain subtypes of gastric cancer may have been excluded from the analyses. This analytical strategy may pronounce the alterations of proteins commonly expressed in gastric cancer.

DDR has been implicated in the tumorigenesis and progression of human cancers (17-19). The impairment of DDR causes the genetic instability of the cell. DDR may serve not only as a barrier to neoplastic cells, but also as a barrier of the progression of neoplastic cells to highly malignant cells (17). The biological significance of DDR in gastric cancer has not yet been fully elucidated. It was previously reported that the expression level of CHK2 was increased in cases with a mutation of the TP53 gene. The expression level of CHK1 was also increased in gastric cancers; however, there was no significant correlation between the expression level of CHK1 and the mutational state of TP53 (20). It has also been demonstrated that the loss of ATM and CHK2 is 17.3 and 12.2% in gastric cancers (21). The percentage of the absence of the expression and/or phosphorylation of CHK2 in gastric cancer in the present study was in agreement with these findings, and its expression and/or phosphorylation were not noted in 6 cases out of the 42 cases (14.3%). Furthermore, the phosphorylation of KU70 appeared to be impaired in one case by immunostaining in the present study.

The impairment of DDR may affect the prognosis of gastric cancer. It has been reported that patients with a positive expression of ATM, CHK2 and TP53 present with a favorable prognosis, and the loss of CHK2 is associated with the reduction of ATM, and/or TP53 is an independent factor for a poor prognosis (21). In the present study, recurrence was noted in 2 out of 7 cases (29%), in which the expression and phosphorylation of DDR-related proteins were impaired, whereas recurrence was noted in 2 out of 35 cases (6%), in which the expression and phosphorylation DDR were intact. The impairment of DDR appeared to be associated with an unfavorable

prognosis. The follow-up duration, and the number of cases was limited in the present study. Further studies are warranted in order to clarify the prognostic significance of DDR.

The aberrant expression of DDR proteins was noted in cultured gastric cancer cell lines. The expression of KU70, ILF2 and CHK2 was aberrant in one cell line, the NS-8 cells, which has characteristic features of the amplification of the *N-ras* gene and the production of α-fetoprotein (22). The consecutive alteration of the expression and phosphorylation of DDR protein following exposure to UV radiation were further examined, and the phosphorylation of CHK1 and CHK2 was decreased in NS-8. The upregulation of TP53 following exposure to UV radiation was noted in only one cell line, NUGC-4, and the increase in apoptotic cell death was evident only in this cell line. It is thus considered that the preservation of DDR and the TP53 response are necessary for the determination of cell fate and the induction of apoptosis. This may in part account for the favorable outcome in patients with gastric cancer with a preserved expression of DDR proteins (21).

DDR may affect the susceptibility to chemotherapy in gastric cancer. The expression of XRCC1 is increased in cisplatin-resistant cancer cells (23). In cases of gastric cancer, the elevated expression of γH2AX and pATM is adverse for progression-free and overall survival (24). In addition, the targeting of CHK2 enhances cell death by treatment with cisplatin and paclitaxel in cultured cell lines (25). It thus appears that the expression of DDR proteins attenuates the susceptibility to chemotherapy, although this may be affected by other genetic factors, such as ARID1A (24). The association of DDR with the efficacy of chemotherapy needs to be elucidated.

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Competing interests

The authors declare that they have no competing interests.

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