Prognostic implications of the DNA damage response pathway in glioblastoma

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Abstract. Genomic instability and resistance to genotoxic therapies for glioblastoma (GBM) suggest aberrant DNA damage response (DDR), since DDR maintains the genomic integrity against genotoxic insults including anti-tumor therapies. To elucidate the biological and clinical meaning of DDR in GBM, we retrospectively investigated the immunohistochemical expression of DDR proteins (ATM, Chk1, Chk2, TopBP1, Rad17, p53, Nbs1, MDC1, γH2AX and RPA1) in 69 GBM surgical samples and their relation with GBM patient survival. Remarkably, higher expression of ATM revealed significantly longer overall survival (OS) and progression-free survival (PFS) ($p<0.05$). Upon multivariate analysis, expression level of ATM was an independent factor for longer OS ($p=0.020$) and longer PFS ($p=0.019$). Since ATM induces cell cycle arrest or apoptosis through cell cycle regulators in response to genotoxic insults, these results indicate that aberrant DDR signaling through ATM in GBM may be associated with resistance to genotoxic anti-tumor therapeutics. Conclusively, we emphasize that the identification of DDR machinery, which can be involved in unstable genomic status or genotoxic therapies in GBM, is very important to predict patient outcome.

Introduction

Glioblastoma (GBM) is the most frequent and malignant human brain tumor with poor response to conventional genotoxic therapies (1). The methylation status of O^6-methylguanine–DNA methyltransferase (*MGMT*) and mutations of isocitrate dehydrogenase (*IDH*) genes have been identified as prognostic and/or predictive biological markers of GBM by comprehensive genetic analyses (2-4). However, the major huddles in treating these tumors still remain, leaving only genotoxic modalities such as radiation and chemotherapy as viable treatment options. To overcome these limitations, novel prognostic markers that could be utilized as therapeutic targets need to be elucidated further.

Cellular DNA damage response (DDR) pathways induce cell cycle arrest and DNA repair in response to the DNA damage (5-7). In many cancers, these DDR machineries are activated by oncogene-evoked replication stress (5,6,8), which might suppress genomic injury induced by genotoxic anti-tumor therapies. Therefore, the DDR machineries could be potential therapeutic targets to overcome the resistance to chemotherapy and radiotherapy in GBM (9,13). Nonetheless, a clinical study on the expression of DDR and the prognostic implication in GBM has yet to be published.

To elucidate the biological and clinical meaning of the DDR pathway in GBM, we present immunohistochemical analyses of DDR or its related proteins such as ataxia telangietactasia mutated (ATM), checkpoint kinase 1 (Chk1), checkpoint kinase 2 (Chk2), topoisomerase binding protein 1 (TopBP1), Rad17, Nijmegen breakage syndrome 1 (Nbs1), mediator of DNA damage checkpoint 1 (MDC1), histone H2A isoform γ (γH2AX), replication protein A 1 (RPA1) and p53. We further report the correlation between the expression level of these components and patient survival.

Materials and methods

Patient and tissue collection. Sixty-nine patients with GBMs were included in this study. Among them, adjacent normal brain tissues were included in 26 cases to compare the differential expressions between GBM and normal tissues. Patient characteristics are summarized in Table I. All patients underwent surgery at our institute between January 2004 and December

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Table I. Demographic findings of the patients.

Characteristics	No.
Patients	69
Gender (male/female)	40/29
Mean age (years)	54.4 (12-79)
Mean F/U periods (days)	562 (51-1558)
Median overall survival (95% CI), days	410 (357-462)
Median progression-free survival (95% CI), days	204 (180-227)
Initial KPS	
≤70	28
>70	41
Location	
Supratentorial	66
Infratentorial	3
Extent of resection	
TR	51
PR	17
Biopsy	1
Adjuvant treatments	
RT only	16
CCRT+TMZ	26
RT+TMZ	27
MGMT status[a]	
Unmethylated	23
Methylated	21

F/U, follow-up; 95% CI, 95% confidence interval; KPS, Karnofsky performance status; TR, total resection; PR, partial resection; RT, radiation therapy; CCRT, concomitant chemoradiotherapy; TMZ, temozolomide chemotherapy. [a]Available tissue samples (N=44) were included.

2006. Patients were managed according to established diagnostic and therapeutic protocols, including surgical resection and subsequent chemoradiotherapy. A macroscopic total resection was performed in 51 of 69 patients (73.8%), a partial resection in 17 of 69 patients (24.6%), and a biopsy only in 1 of 69 patients (1.4%). All patients underwent subsequent radiotherapy (60 Gy in 2 Gy fractions) after surgery. For concurrent chemoradiotherapy or adjuvant chemotherapy, 53 of 69 patients received temozolomide with a median of 4 cycles (range, 1-9 cycles). The remaining 16 patients (23.2%) did not receive chemotherapy because of clinical deterioration during radiotherapy. Tumor samples were re-evaluated by two neuropathologists to confirm the diagnosis according to the World Health Organization criteria. Tumor samples were obtained during surgical treatment and were embedded in paraffin for histological studies. Written informed consent was obtained from all patients, and tissue collection was approved by the institutional review board. Immunohistochemical (IHC) studies were performed in a double-blinded manner, without prior knowledge of clinical outcome.

Immunohistochemical study. Four-micrometer-thick sections sliced from paraffin-embedded specimens were prepared on the slide. Sections were immunostained with antibodies for ATM (1:50, Santa Cruz Biotechnology, CA, USA), Chk1 (1:50, Santa Cruz Biotechnology), Chk2 (1:50, Santa Cruz Biotechnology), TopBP1 (1:50, Bethyl Laboratories, Inc., Montgomery, TX, USA), Rad17 (1:50, Santa Cruz Biotechnology), p53 (1:5000, Santa Cruz Biotechnology), Nbs1 (1:500, GeneTex, Irvine, CA, USA), MDC1 (1:250, Bethyl Laboratories, Inc.), γH2AX (1:500, Abcam, Cambridge, MA, USA), RPA1 (1:100, Calbiochem, San Diego, CA, USA), p21 (1:40, Lab Vision, Fremont, CA, USA), p18 (1:20, Lab Vision), MDM2 (1:100, Lab Vision) and p27 (1:40, Lab Vision). Tumor-containing sections were baked at 56˚C for 30 min, deparaffinized in xylene and rehydrated in graded concentrations of ethanol. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol and with heat-induced antigen retrieval [for p53, 10 mM citrate buffer (pH 6.0) for 25 min in a vegetable steamer]. Immunostaining involved sequential applications of primary antibody for 16 h at 4˚C, followed by biotinylated secondary antibodies (Vector Laboratories, Orton Southgate, UK) at 1:200 for 1 h and avidin (Elite ABC; Vector Laboratories) for 1 h. Negative control slides received normal horse and goat serum (Dako Corp., Carpinteria, CA, USA) as the primary antibody. Diaminobenzidine tetrahydrochloride was used as the enzyme substrate to observe the specific antibody localization, and Harris hematoxylin was used as a nuclear counterstain.

Sections were examined for immunoreactivity for the proteins by an observer who was unaware of the pathological diagnoses, outcomes or clinical features. Tumors were categorized as follows: Grade 0 for those expressing no protein, Grade 1 for those expressing in <25% of cells, Grade 2 for those expressing in 25-50% of cells at similar or overexpressed levels compared to the normal brain, Grade 3 for those in 50-75% of cells and Grade 4 for those in >75% of cells, based on the expression level in nucleus visualized in a high-power field in areas with maximal staining. The expression of the proteins was analyzed as a dichotomous covariate: no or little immunoreactivity (Grade 0 or 1) vs. overexpression (Grades 2-4).

Bisulfite modification and methylation-specific PCR. DNA (1 μg) from the tumor was denatured by sodium hydroxide and modified by sodium bisulfite. Methylation-specific PCR was performed as previously described (14).

Cell culture and cell lines. Human glioma cell lines, U138MG, U373MG, U87MG, U251MG and control human osteosarcoma cells (U2OS) (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in a 37˚C, 5% CO_2 humidified chamber.

Ionizing radiation and Western blotting. Cells were lysed and prepared for Western blot analysis as previously described (15). Cells were processed 6 h after ionizing radiation (IR) (10 Gy), washed (PBS) and harvested. Anti-ATM, -ATR, -Chk1 anti-

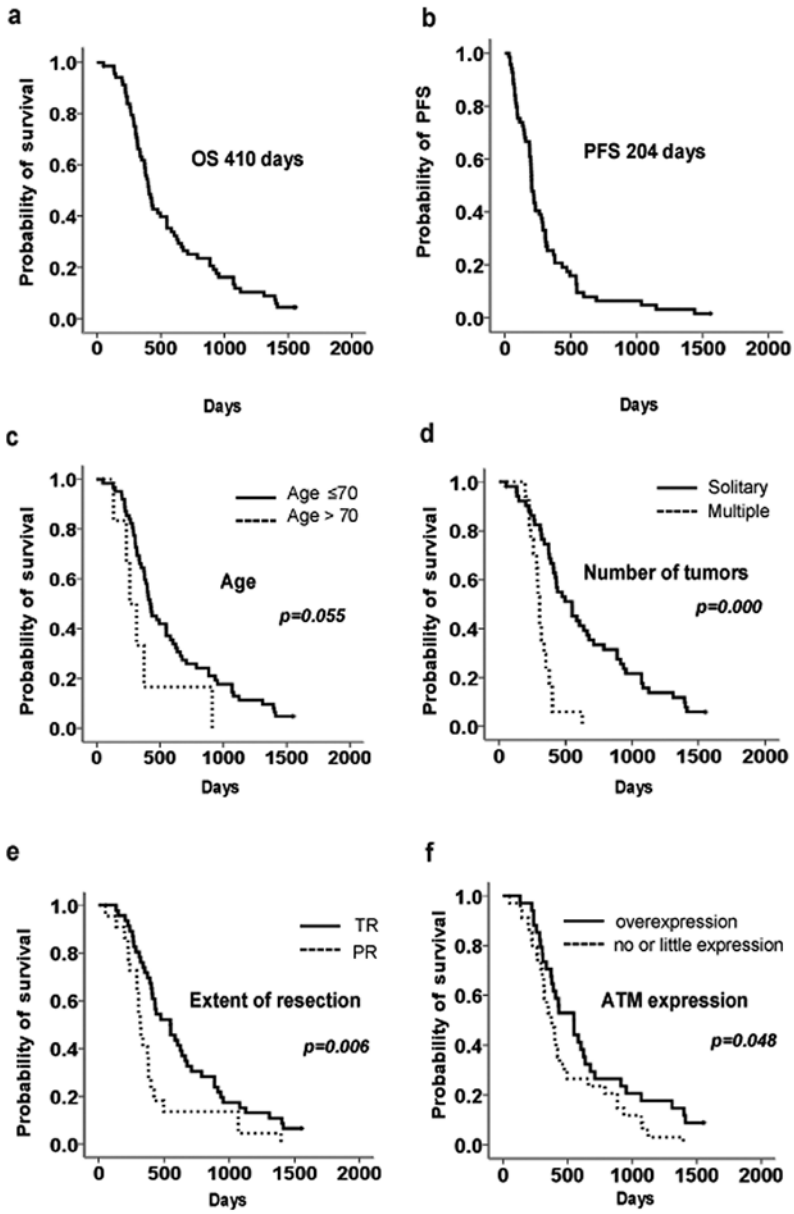

Figure 1. Overall survival (OS) (a) and progression-free survival (PFS) (b) of 69 glioblastoma patients. OS curve according to age (c), number of tumors (d) and extent of resection (e) demonstrated significance ($p<0.05$) or borderline significance (p-value of the age factor showed borderline significance). Remarkably, ATM overexpressing patients showed longer survival ($p=0.048$) (f).

bodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology). Anti-TopBP1 and -MDC1 were obtained from Bethyl Laboratoratoies. Anti-human α-tubulin antibody was from Oncogene Research Products (Cambridge).

Statistical analysis. The comparison of expression levels between tumors and adjacent normal brain was analyzed by paired sample t-test. The analysis of the relation between the above protein expressions and overall or progression-free survival was investigated by using the Kaplan-Meier method (SPSS statistical software, version 18.0; SPSS Inc., Chicago, IL, USA). The differences between the survival curves were tested using the log-rank test. The correlation among the variables was analyzed by Spearman correlation coefficient. The multivariate survival analysis was performed according to the Cox proportional hazards model in a forward stepwise manner. The results are reported as statistically significant at 2-sided $p<0.05$. The Fisher's exact test was used to analyze the correlation between the IHC variables.

Results

Clinical characteristics of 69 GBM patients. The mean age of the 69 GBM patients included in this study was 54.4 (range, 12-79) years. There were 40 men and 29 women. The mean follow-up period was 562 (51-1558) days, and the median overall survival was 410 [95% confidence interval (95% CI), 357-462] days (Fig. 1a). The median progression-free survival was 204 (95% CI, 180-227) days (Fig. 1b). Other demographic data are described in Table I.

Table II. Univariate analysis of survival.

Prognostic factors	No.	Median OS, days	p-value[a]	Median PFS, days	p-value[b]
Age, (years)					
≤70	62	422	0.055	204	0.948
>70	7	262		262	
Gender					
Male	40	378	0.124	204	0.950
Female	29	431		202	
KPS					
≤70	28	401	0.427	204	0.305
>70	41	410		221	
No. of tumors					
1	52	548	0.000	289	0.502
>1	17	304		362	
Extent of resection					
TR	51	548	0.006	217	0.214
PR	18	316		201	
Status of MGMT					
Unmethylated	23	407	0.628	199	0.759
Methylated	21	410		217	
Expression of ATM					
No or little expression	34	373	0.048	196	0.029
Overexpression	35	548		278	
Expression of Chk1					
No or little expression	23	399	0.848	204	0.757
Overexpression	46	410		204	
Expression of Chk2					
No or little expression	46	379	0.091	203	0.555
Overexpression	22	549		204	
Expression of Rad17					
No or little expression	42	410	0.958	205	0.910
Overexpression	26	407		189	
Expression of TopBP1					
No or little expression	20	318	0.076	203	0.723
Overexpression	49	474		204	
Expression of p53					
No or little expression	54	407	0.230	201	0.004
Overexpression	15	583		471	
Expression of Nbs1					
No or little expression	18	320	0.579	262	0.593
Overexpression	51	410		202	
Expression of MDC1					
No or little expression	26	401	0.741	199	0.521
Overexpression	42	410		204	
Expression of γH2AX					
No or little expression	22	422	0.587	204	0.900
Overexpression	46	407		203	
Expression of RPA1					
No or little expression	42	422	0.812	217	0.397
Overexpression	26	379		188	

OS, overall survival; PFS, progression-free survival; KPS, Karnofsky performance status; TR, total resection; PR, partial resection.

Table III. Comparative expression of DDR protein in 26 paired GBMs and normal brains.

DDR proteins	Mean ± standard deviation	p-value
ATM-T/ATM-N	2.19±1.29/1.62±0.88	0.032
Chk1-T/Chk1-N	2.15±1.37/1.58±1.13	0.029
Chk2-T/Chk2-N	1.46±1.42/0.85±0.54	0.018
TopBP1-T/TopBP1-N	2.85±1.08/2.12±0.86	0.003
Rad17-T/Rad17-N	2.12±1.33/2.12±1.03	1.000
p53-T/p53-N	0.92±1.38/0.23±0.71	0.017
Nbs1-T/Nbs1-N	2.58±1.02/1.46±0.64	0.000
MDC1-T/MDC-1-N	2.96±1.11/3.12±0.95	0.557
γH2AX-T/γH2AX-N	2.53±1.14/2.81±1.02	0.099
RPA1-T/RPA1-N	1.12±1.07/0.81±0.80	0.212

-T, the expression level in tumor; -N, the expression in adjacent normal brain.

Table IV. Multivariate analysis of OS and PFS.

Prognostic factors	Hazard ratio (95% CI)	p-value
OS		
Age >70	2.761 (1.064-7.166)	0.037
Multiple masses	4.202 (2.136-8.267)	0.000
Total resection	0.631 (0.344-1.156)	0.136
Expression of ATM	0.530 (0.310-0.904)	0.020
Expression of TopBP1	0.858 (0.437-1.684)	0.657
Expression of p53	0.713 (0.327-1.556)	0.395
PFS		
Expression of ATM	0.525 (0.307-0.898)	0.019
Expression of p53	0.413 (0.209-0.818)	0.011

95% CI, 95% confidence interval.

Univariate analysis of possible prognostic factors revealed several meaningful results that were related to overall survival (OS) or progression-free survival (PFS) (Table II, Fig. 1a and b). Age factor showed borderline significance as follows: patients >70 years of age (n=7) showed lower median survival than those ≤70 years of age (n=62) (p=0.055, Fig. 1c). However, the factor was not found to be correlated with PFS (p=0.948). Gender, preoperative Karnofsky performance status (KPS), and the methylation status of MGMT were not correlated with patient OS or PFS (Table II). Solitary tumors (n=52) showed significantly longer OS than multiple tumors (n=17) (p<0.001, Fig. 1d). In addition, total resection (n=51) revealed better OS than partial resection or biopsy (n=18) (p=0.006, Fig. 1e).

Expression of components of the DDR pathway in GBM. Expression of 10 DDR-related proteins (ATM, Chk1, Chk2, TopBP1, Rad17, p53, Nbs1, MDC1, γH2AX and RPA1) was analyzed immunohistochemically. Expression level of each protein was categorized as follows: Grade 0 for those expressing no protein, Grade 1 for those expressing in <25% of cells, Grade 2 for those expressing in 25-50% of cells, Grade 3 for those in 50-75% of cells and Grade 4 for those in >75% of cells.

At first, alteration in the expression of the 10 DDR-related proteins in GBM was analyzed using 26 GBM and paired adjacent normal brain samples. Expression of all the 10 DDR-related proteins was increased in GBM compared with relatively normal brain (Table III). Especially, ATM, Chk1, Chk2, TopBP1, p53 and Nbs1 were overexpressed significantly in GBMs (p<0.05, Table III). Therefore, expression of components of the DDR pathway is maintained or increased in GBM.

Clinical and prognostic implications of the DDR pathway in GBM. To find out the clinical implication of increased expression of the DDR pathway, 69 GBM patients were classified into two groups for each DDR-related protein; no or little immunoreactivity (Grade 0 or 1) vs. overexpression (Grades 2-4) (Table II). When OS and PFS were compared between the two groups for each DDR-related protein by univariate analysis, the expression of ATM showed significant correlation with both OS and PFS of the patients (p=0.048 and 0.029, respectively, Table II, Fig. 1f). Higher expression level of this protein revealed longer survival. Higher expression of p53 was also associated with longer PFS (p=0.004). Other DDR proteins were not correlated with OS or PFS, although borderline significance was observed with TopBP1 in OS (p=0.076).

On multivariate analysis (Table IV), age >70 years and presence of multiple masses were significant prognostic factors of poor OS (p=0.037 and 0.000, in each). Remarkably, higher expression of ATM was a significant independent factor for longer OS (p=0.020, Table IV). For longer PFS, the increased expression of ATM and p53 were independent prognostic factors (p=0.019 and 0.011, respectively, Table IV) confirming prognostic significance of expression of ATM in GBM.

Correlation of expression between cell cycle regulators and ATM. Since DDR pathways induce cell cycle arrest in response to DNA damage (5-7), ATM overexpression in GBM might be related with the alteration in the regulatory mechanism of cell cycle. Therefore, p21, p27, p18 and MDM2 were additionally immunostained in 69 GBM patients, expression level of each protein was categorized using the same method with DDR proteins, and correlation with ATM expression was analyzed. The Spearman correlation coefficients (p-value) are as following: p53, 0.244 (0.043); p27, 0.339 (0.006); p21, 0.240 (0.056); p18, 0.107 (0.398); and MDM2, 0.213 (0.086). Resultantly, expression level of ATM was positively correlated with that of p53, p27 and p21. The representative IHC results of correlation among ATM, p53, p21 and p27 are presented in Fig. 2.

Results in this study indicate that maintained or increased expression of ATM and related cell cycle regulators suggest better clinical outcomes of GBM patients (OS and PFS). Since ATM induces cell cycle arrest or apoptosis in response to genotoxic insults such as irradiation, these results also

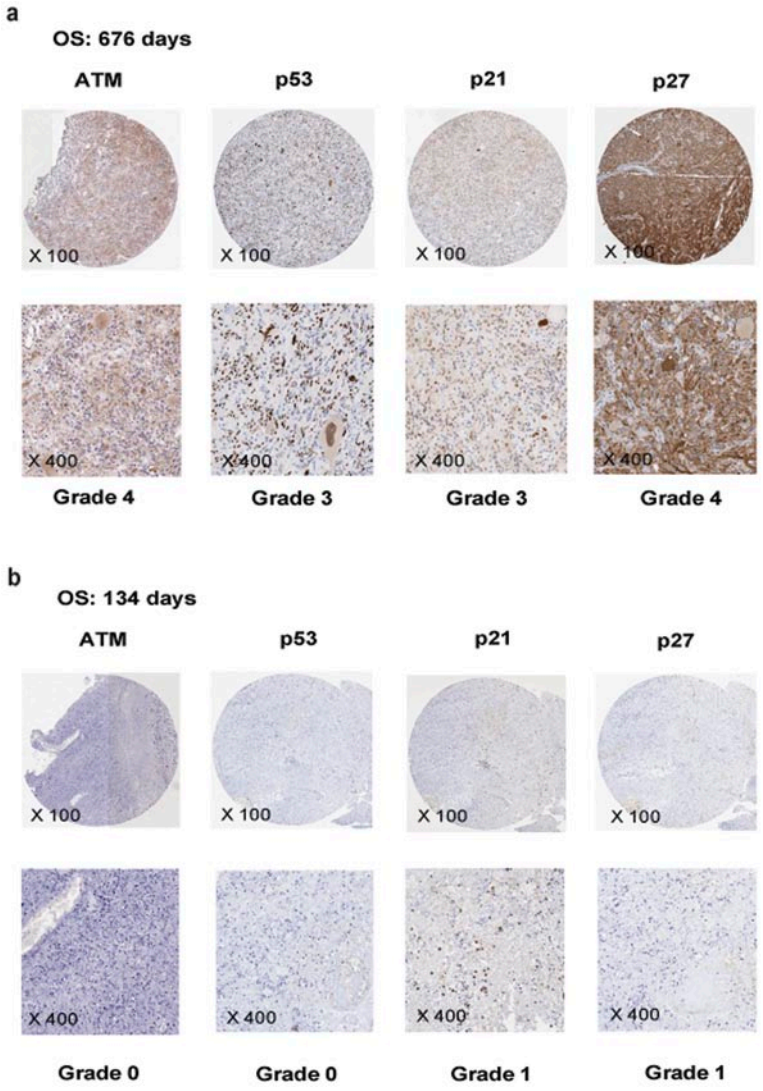

Figure 2. Representative immunohistochemical analysis with tissue microarray showed strong relationship between overall survival (OS) and level of ATM expression. The higher expression level of ATM associated with p53, p21 and p27 showing longer OS (a). Strong inverse relationship between OS and expression level of ATM was observed by immunohistochemical analysis (b).

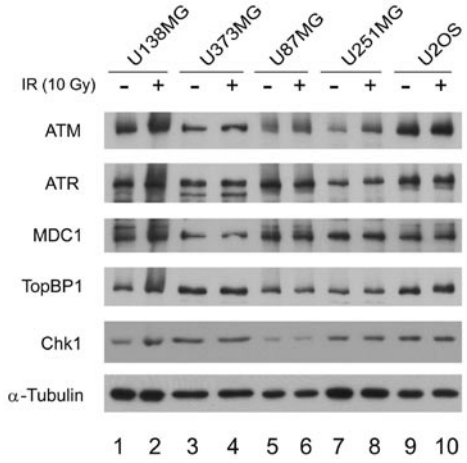

Figure 3. DNA damage response (DDR) is constitutively activated in glioma cell lines after irradiation. The level of expression was compared before and 6 h after IR (10 Gy). Varying degrees of basal expression for ATM, ATR, MDC1, TopBP1 and Chk1 are shown. The expression of ATM was slightly increased after IR in U138MG and U251MG. U2OS, human osteosarcoma cell; IR, ionizing radiation.

indicate that maintained signaling through ATM and downstream cell cycle regulator might be needed for therapeutic effects of genotoxic anti-tumor modalities.

Alteration in ATM expression of glioma cells in response to irradiation. In the response to DNA damage, several component of DDR pathway such as ATM, ATR, MDC1, TopBP1 and Chk1 are involved in the initiation of signaling. Above results indicate that ATM could be the major initiator of signaling and induce cell cycle arrest and/or apoptosis in response to genotoxic insults in GBM. We analyze alteration in the expression levels of DDR proteins (ATM, ATR, MDC1, TopBP1 and Chk1) in glioma cell lines (U138MG, U373MG, U87MG, U251MG) and U2OS, 6 h after 10 Gy *in vitro* irradation (Fig. 3). The cell lines showed varying degrees of basal expression for ATM, ATR, MDC1, TopBP1 and Chk1. The level of expression was similar or slightly increased after *in vitro* irradation. However, interestingly, the expression of ATM was increased after irradiation in U138MG and U251MG. These data suggest that ATM and its downstream

signaling components affected the response to irradiation in GBM.

Discussion

Numerous studies have revealed cascades of DDR after DNA damage such as IR or genotoxic therapies (16,19). However, they have been mainly focused on the signal pathway and molecular interactions. There has been no report suggesting the clinical implication of DDR in GBM. Therefore, we performed this study to examine the prognostic meaning of DDR in GBM.

In this study, the samples were mostly removed before radiation or chemotherapy. The expression of DDR proteins including ATM was higher in tumor samples than in their adjacent normal samples. Thus, these results indicated that the DDR machinery became activated because of some aberrant, stressful endogenous processes that threaten the genomic integrity and/or occur during the constitutive development of GBM (5,7). In this study, ATM was revealed to be an independent prognostic factor related to longer OS and PFS. p53 was also related to longer PFS. Higher expression of TopBP1, which is a direct activator of ATR (20) and a connector of ATM to ATR (17), revealed borderline significance for longer OS. In addition, we showed that the expression level of ATM was positively correlated with that of p27 and p21 as well as p53. Since DDR pathways are related with the alteration in the regulatory mechanism of cell cycle (5-7), the tumor suppressors p21 and p27 might be functionally-correlated with p53 in many cancers (21). Moreover, increased p21, which is a downstream protein of p53, is thought to be an indicator of intact p53 (21,22). There are many studies suggesting that lower expression of p27 and p21 could be a negative prognostic marker in various cancers (23-25). Therefore, our positive prognostic results of ATM expression in conjunction with tumor suppressor genes such as p53, p21 and p27 might be explained in GBMs.

DDR machinery including ATM is thought to be a barrier against glioma progression, because it may limit the expansion of nascent malignant clones with unstable genome (5,6). Furthermore, Gilbert and Hemann (26) suggested ATM inhibition could result in increasing secretion of cytokines such as IL-6, which is an important factor secreted from the endothelial cells around chemoresistant niche. It may also explain the role of ATM for increasing efficacy of genotoxic therapies in some tumors. On the other hand, some researchers have suggested the possibility of DNA damage checkpoints or repair inhibitors for radiosensitizing in glioma therapies (9-13,27-29). Their experimental data suggested DDR machinery could be a target to overcome the resistance to chemotherapy and radiotherapy in GBM, because DNA repair systems were transiently inhibited after administration of inhibitors for DDR.

On the basis of our clinical analysis in conjunction with previous reports (1,5,6), further clinical trials targeting DDR in GBM should be very cautiously approached. It is because DDR can be a continuous defensive process even in malignant tumors, which might be a reflection of favorable outcome. In addition, maintaining the DDR signaling through ATM and downstream cell cycle regulator might be needed for therapeutic effects of genotoxic anti-tumor modalities, considering the original role of ATM for cell cycle arrest or apoptosis. Even though further larger scaled studies are needed, we emphasize that the identification of DDR machinery, which can be involved in unstable genomic status or genotoxic therapies in GBM, is very important to predict patient outcome.

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