

# $\gamma$ -H2AX as a potential indicator of radiosensitivity in colorectal cancer cells

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**Abstract.** Preoperative radiotherapy improves local disease control and disease-free survival in patients with advanced rectal cancer; however, a reliable predictive biomarker for the effectiveness of irradiation has yet to be elucidated. Phosphorylation of H2A histone family member X (H2AX) to  $\gamma$ -H2AX is induced by DNA double-strand breaks and is associated with the development of colorectal cancer (CRC). The current study aimed to clarify the relationship between  $\gamma$ -H2AX expression and CRC radiosensitivity *in vitro* and *in vivo*. H2AX levels were analyzed in datasets obtained from cohort studies and  $\gamma$ -H2AX expression was investigated by performing immunohistochemistry and western blotting using clinical CRC samples from patients without any preoperative therapy. In addition, the CRC cell lines WiDr and DLD-1 were subjected to irradiation and/or small interfering RNA-H2AX, after which the protein levels of  $\gamma$ -H2AX were examined in samples obtained from patients undergoing preoperative chemoradiotherapy. To quantify the observable effect of treatment on cancer cells, outcomes were graded as follows: 1, mild; 2, moderate; and 3, marked, with defined signatures of cellular response. Datasets obtained from cohort studies demonstrated that H2AX mRNA levels were significantly upregulated and associated with distal metastasis and microsatellite instability

in CRC tissues, in contrast to that of normal tissues. In addition,  $\gamma$ -H2AX was overexpressed in clinical samples. *In vitro*, following irradiation,  $\gamma$ -H2AX expression levels increased and cell viability decreased in a time-dependent manner. Combined irradiation and  $\gamma$ -H2AX knockdown reduced the viability of each cell line when compared with irradiation or  $\gamma$ -H2AX knockdown alone. Furthermore, among clinical CRC samples from patients undergoing preoperative chemoradiotherapy, levels of  $\gamma$ -H2AX in the grade 1 group were significantly higher than those in grade 2 or grade 3. In conclusion,  $\gamma$ -H2AX may serve as a novel predictive marker and target for preoperative radiotherapy effectiveness in patients with CRC.

## Introduction

Colorectal cancer (CRC) is one of the most common human cancers and is associated with high morbidity and mortality (1). Particularly in cases of advanced rectal cancer, preoperative radiotherapy (PRT) and chemoradiotherapy (PCRT) improve local control and long-term disease-free survival, compared with surgery alone (2,3). Furthermore, PCRT has been one of the standard therapies for rectal cancer regarding anus preservation and is used for the prevention of local recurrence and presurgical downstaging (4). In practice, the efficiency of PCRT varies between individuals; pathologic complete response has been reported in the range of 15 to 20% (5-7). Various clinical factors have been reported as predictors of histological regression or tumor downstaging in rectal cancer, such as the circumferential extent of the tumor, distance from the anal verge, and serum levels of carcinoembryonic antigen (CEA) (8-10). However, the validation of these predictors remains insufficient.

The phosphorylation of histone H2AX (into  $\gamma$ -H2AX) is induced by DNA double-strand breaks (DSB). As tumor cells are usually deficient in DNA damage response (DDR) pathways, it has been suggested that constitutive expression of histone  $\gamma$ -H2AX might indicate the disruption of DDR pathways and genomic instability (11).  $\gamma$ -H2AX expression gradually, but significantly, increases during tumor progression in human CRC (12). *In vitro*, increasing levels of  $\gamma$ -H2AX, after irradiation, have been correlated with radiosensitivity in 18 human cell

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**Abbreviations:** CRC, colorectal cancer; DDR, DNA damage response; DMSO, dimethyl sulfoxide; DSB, double-strand breaks; IHC, immunohistochemistry; PARP, poly (ADP-ribose) polymerase; PCRT, preoperative chemoradiotherapy; siRNA, small interfering RNA

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lines (13). Additionally, high  $\gamma$ -H2AX expression is associated with poor prognosis in CRC patients (14). Therefore, we hypothesized that the expression level of  $\gamma$ -H2AX is a predictor of radiosensitivity in rectal cancer. In this study, we sought to clarify the relationship between  $\gamma$ -H2AX expression and radiosensitivity in CRC, using *in vivo* and *in vitro* experiments.

## Materials and methods

**Data set analysis.** The OncoPrint™ Research Platform (Thermo Fisher Scientific, Inc.) was used in this study. The mRNA expression levels of H2AX were investigated in each cohort study. The detailed methodology of these studies is available in the references (15-22).

**Cell culture.** Human CRC cell lines WiDr and DLD-1 were purchased from JCRB (Japanese Collection of Research Bio Resources) Cell Bank. Both lines were authenticated by short tandem repeat (STR) sequence profiling by JCRB. STR examination showed that the WiDr was identical to HT-29 (23). All cells were cultured in RPMI-1640 (HyClone; GE Healthcare Life Sciences) supplemented with 10% (v/v) heat-inactivated fetal bovine serum at 37°C, in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Patient samples.** Six pairs of CRC tissues and adjacent normal mucosa tissues, and eleven pairs of endoscopic biopsy samples from CRC tissues and adjacent normal mucosa, were obtained from patients who had undergone surgical resection of their tumor between 2013 and 2015 at Osaka Medical College Hospital (Takatsuki, Osaka, Japan). Collection and investigation of the samples were approved by the research Ethics Committee of Osaka Medical College (approval no. 1280, 2 September 2013) in accordance with the Declaration of Helsinki. Before treatment, each patient provided written, informed consent regarding the use of their tissues in our research. All tissue sample pairs were collected from the same patient. Detailed clinical information is shown in Tables I and II. Pathological staging of the cancers was performed according to postoperative pathological reports, using guidelines for the treatment of colorectal cancer from the Japanese Society for Cancer of the Colon and Rectum 2010 (24). Each 'grade of effect', induced by PCRT, was evaluated histologically by our hospital's pathologist, using surgically resected specimens. The criteria for the assessment of response to PCRT are defined as follows: Grade 0 (no effect): No tumor cell necrosis or degeneration was observed. Grade 1 (mild effect): Tumor cell necrosis or degeneration is present in less than one third of the entire lesion (minimal effect) or in more than one third but less than two thirds of the entire lesion (mild effect). Grade 2 (moderate effect): Although prominent tumor cell necrosis, degeneration, lytic change, and/or disappearance is present in more than two thirds of the entire lesion, viable tumor cells remain. Grade 3 (marked effect): Necrosis and/or lytic change is present throughout the entire lesion, accompanied by replacement of fibrosis, and viable tumor cells were not observed. Assessment was performed on as many pathological specimens as possible, including those prepared from the section of the whole tumor at the point of maximum diameter (25).

**Irradiation time course experiments.** CRC cells were seeded in 6-well plates at a concentration of  $0.4 \times 10^4$  cells per well (10-30% confluence) the day before irradiation. After irradiation at 10 Gy, cells were incubated for 24, 48, 72 and 96 h, and the effects were assessed.

**Gene silencing in irradiation experiments.** siRNA (siR) for H2AX was purchased from Santa Cruz Biotechnology. Silencer negative control siRNA (Invitrogen; Thermo Fisher Scientific, Inc.) was used as a control for nonspecific effects. Cells were transfected with 10 nM siRNAs using Lipofectamine™ RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 24 h of transfection, the cells were irradiated with 10 Gy and subsequently collected after 72 h.

**Cell viability.** MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) solution was purchased from Sigma-Aldrich; Merck KGaA. The detailed protocol is described in a previous report (26). Absorbance at 540 nm was measured using an SH-1000 Lab microplate reader (Corona Electric Co., Ltd.).

**Immunohistochemistry (IHC).** Detailed protocol information is described in our previous reports (26,27). Anti-phospho-histone H2A.X (Ser139) antibody (EMD Millipore) was used. Images were taken with a BZ-x700 microscope (Keyence Co.).

**Western blot analysis.** Detailed protocol information is described in our previous reports (26-28). The primary antibodies used were as follows: anti-phospho-histone H2A.X (Merck Millipore; 1:1,000) and anti- $\beta$ -actin (Cell Signaling Technology, Inc.; 1:1,000). HRP-conjugated horse anti-mouse and anti-rabbit IgGs (Cell Signaling Technology; 1:1,000) were used as secondary antibodies. Immunoblots were detected and visualized using Fusion-FX7 (Vilber Lourmat).

**Statistical analysis.** Each experiment was performed in triplicate. The data are presented as the mean  $\pm$  SE. All statistical analyses were performed using JMP® 12 (SAS Institute Inc.). Statistical differences between the mean values of multiple groups were determined using analysis of variance followed by Student's t-test or one-way analysis of variance (ANOVA). Tukey-Kramer test was performed post hoc following one-way ANOVA. P-values <0.05 indicated statistical significance.

## Results

**Significant upregulation of H2AX mRNA in CRC tissues.** The expression of  $\gamma$ -H2AX is increased in advanced CRC and is associated with poor prognosis (12,14). First, the mRNA levels of H2AX were investigated using datasets from cohort studies. Our dataset analysis showed that the mRNA levels of H2AX were significantly upregulated in CRC tissues compared to those in normal tissues, except for one cohort study (Fig. 1A). The expression levels of H2AX in CRC patients with distant metastasis (M1 Primary), including liver metastasis (Liver Met), was higher than that in patients without metastasis (M0 Primary; Fig. 1B). Interestingly, H2AX mRNA levels in the group positive for microsatellite instability (MSI) were higher than those

Table I. Clinical and pathological features of patients with CRC without any preoperative therapy.

Case	Age	Sex	Location	Type	Tumor diameter (mm)	Pathology	Tumor <sup>a</sup>	Node <sup>a</sup>	Metastasis <sup>a</sup>	Stage <sup>a</sup>
1	62	M	A	1	23x18	tub1	2	0	0	I
2	62	M	S	2	64x38	tub2, tub1	3	0	0	IIA
3	58	M	R	2	53x50	tub1=pap>tub2	3	1a	1	IV
4	67	M	T	3	56x54	tub2>tub1	3	1a	1	IV
5	68	F	R	2	54x44	tub2, tub1>por2	3	1a	0	IIIB
6	58	M	R	0	15x12	tub1, tub2	1	0	0	I

<sup>a</sup>Clinical stage according to UICC TMN classification of malignant tumors (eighth edition) (24). M, male; F, female; A, ascending colon; S, sigmoid colon; T, transverse colon; R, rectum; type 1, mass type; type 2, localized ulcerative type; type 3, infiltrative ulcerative type; pap, papillary adenocarcinoma; tub1, well-differentiated tubular adenocarcinoma; tub2, moderately differentiated; por1, poorly differentiated adenocarcinoma (solid type); por2, (non-solid type).

Table II. Clinical and pathological features of patients with rectal cancer receiving preoperative chemoradiotherapy.

Case	Age	Sex	Type	Pathology	Tumor <sup>a</sup>	Node <sup>a</sup>	Metastasis <sup>a</sup>	Stage <sup>a</sup>
A, Grade 1a + 1b								
1	41	M	2	tub1, tub2	3	1	0	IIIB
2	77	M	3	tub1, tub2	3	1	0	IIIB
3	62	F	3	tub2	3	0	0	IIA
B, Grade 2								
1	72	M	3	tub1, tub2	3	1	0	IIIB
2	67	F	2	tub1	3	1	0	IIIB
3	74	M	3	tub2	3	0	0	IIA
4	62	M	2	tub2>tub1	3	0	0	IIA
C, Grade 3								
1	57	M	2	tub2>por	3	2a	0	IIIB
2	65	M	2	tub1	3	0	0	IIA
3	67	M	2	tub2	3	1	0	IIIB
4	71	M	2	tub1, pap	3	0	0	IIA

<sup>a</sup>Pathological stage determined according to the UICC TNM classification of malignant tumors (eighth edition) (24). M, male; F, female; type 2, localized ulcerative type; type 3, infiltrative ulcerative type; pap, papillary adenocarcinoma; tub1, well-differentiated tubular adenocarcinoma; tub2, moderately differentiated; por, poorly differentiated adenocarcinoma.

in the MSI-negative group (Fig. 1C). These results suggest that upregulation of H2AX mRNA is associated with the development of CRC, in a similar way to the expression of  $\gamma$ -H2AX.

*The protein expression of  $\gamma$ -H2AX was upregulated in CRC specimens compared to adjacent normal tissues.* We examined the protein expression of  $\gamma$ -H2AX in advanced CRC tissue without preoperative therapy (Table I). IHC showed that almost all nuclei in cancer cells were stained, indicating strong expression of  $\gamma$ -H2AX compared to that in adjacent normal tissues (Fig. 2A). The same tendency was observed during western blot analysis (Fig. 2B and C). These findings support the results of a previous study (12), indicating that the expression levels of  $\gamma$ -H2AX were upregulated in advanced CRC tissue.

*The suppression of  $\gamma$ -H2AX facilitated the inhibition of cell viability induced by irradiation in CRC cells.* To assess the

protective effect of  $\gamma$ -H2AX against irradiation, changes in the expression levels of  $\gamma$ -H2AX were measured after irradiation treatment, in two CRC cell lines. As shown in Fig. 3A and B, the expression level of  $\gamma$ -H2AX increased in correlation with irradiation-induced inhibition of cell viability, in a time-dependent manner. Subsequently, we examined the effects of combination treatment (irradiation and knockdown of H2AX by siRNA) in these cell lines. As expected, additional inhibition of growth was observed with the combination therapy, compared to that with irradiation or siR-H2AX alone, in both cell lines (Fig. 3C and D). These results imply that the expression of  $\gamma$ -H2AX is associated with radiosensitivity in CRC cells.

*Sensitivity to preoperative chemoradiotherapy was enhanced in the low  $\gamma$ -H2AX-expression group of patients with advanced rectal cancer.* Finally, we investigated the role of

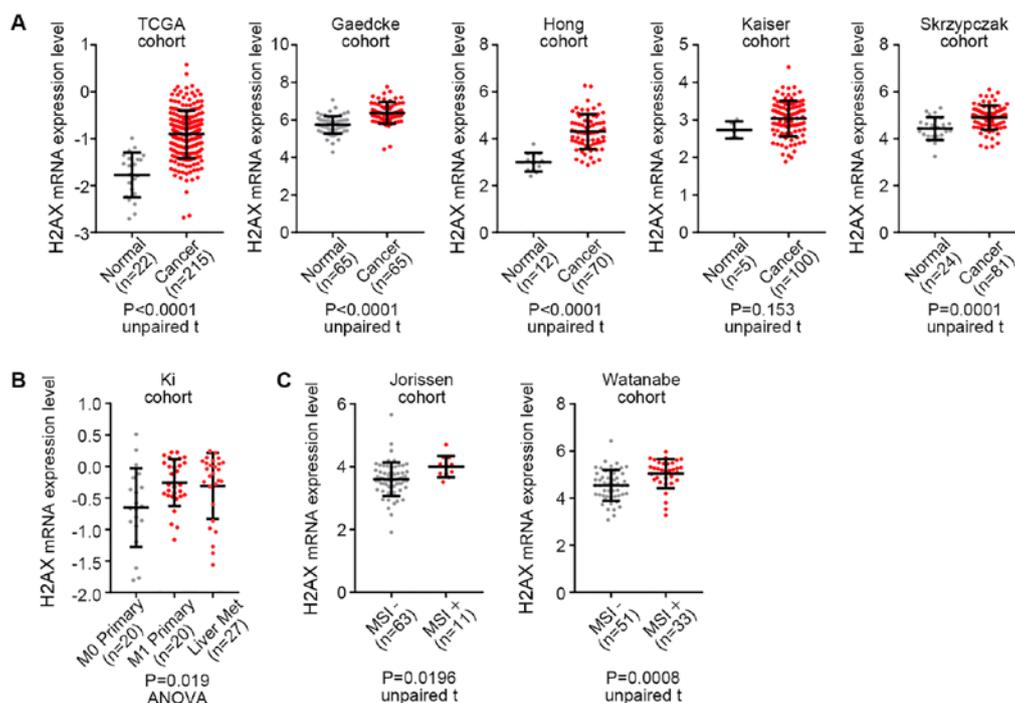


Figure 1. mRNA expression profiles of H2AX in several CRC cohort studies. (A) mRNA levels of the five CRC cohort studies. (B) H2AX mRNA levels of patients with M1 primary or liver Met compared with M0 primary. (C) H2AX mRNA levels in positive and negative MSI groups. H2AX, H2A histone family member X; CRC, colorectal cancer; M1 primary, distant metastases; liver Met, liver metastases; M0 primary, non-metastasis; MSI, microsatellite instability; TCGA, The Cancer Genome Atlas.

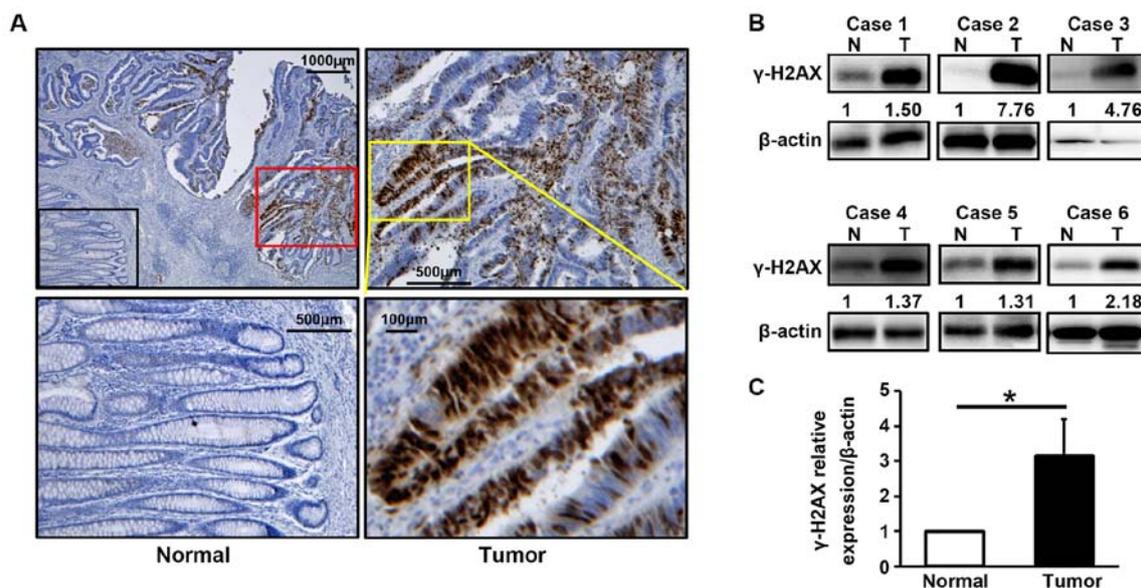


Figure 2. Protein expression of  $\gamma$ -H2AX in rectal cancer tissues. (A)  $\gamma$ -H2AX expression in rectal cancer tissues was measured via IHC staining. The IHC results of a representative case are presented. The upper left panel indicates the overall cancer boundary area; the lower left panel demonstrates an enlarged image of the normal area (black box in overall cancer boundary image); the upper right panel indicates the tumor area (red box in overall cancer boundary image); and the lower right panel indicates an enlarged image of the yellow box in the upper left panel. Scale bars are provided in each image. (B)  $\gamma$ -H2AX protein expression in six randomly selected rectal cancer tissues were determined via western blot analysis. (C) Quantification of results in (B). Values represent the mean  $\pm$  standard error. \*P < 0.05 as indicated. H2AX, H2A histone family member X; IHC, immunohistochemistry; N, normal; T, tumor.

$\gamma$ -H2AX *in vivo*. We examined the response to PCRT in biopsy samples, based on preoperative inspection (Table II). As shown in Fig. 4A and B, the expression levels of  $\gamma$ -H2AX in grade 2 or grade 3 tissues were significantly lower than those of the grade 1 group. These results suggest that low expression of  $\gamma$ -H2AX enhances radiosensitivity in CRC cells (Fig. 4C).

## Discussion

In this study, we found that the mRNA levels of H2AX and the expression of  $\gamma$ -H2AX in CRC tissue were both higher than in normal tissues.  $\gamma$ -H2AX has also been reported as a diagnostic and prognostic marker in other types of cancer,

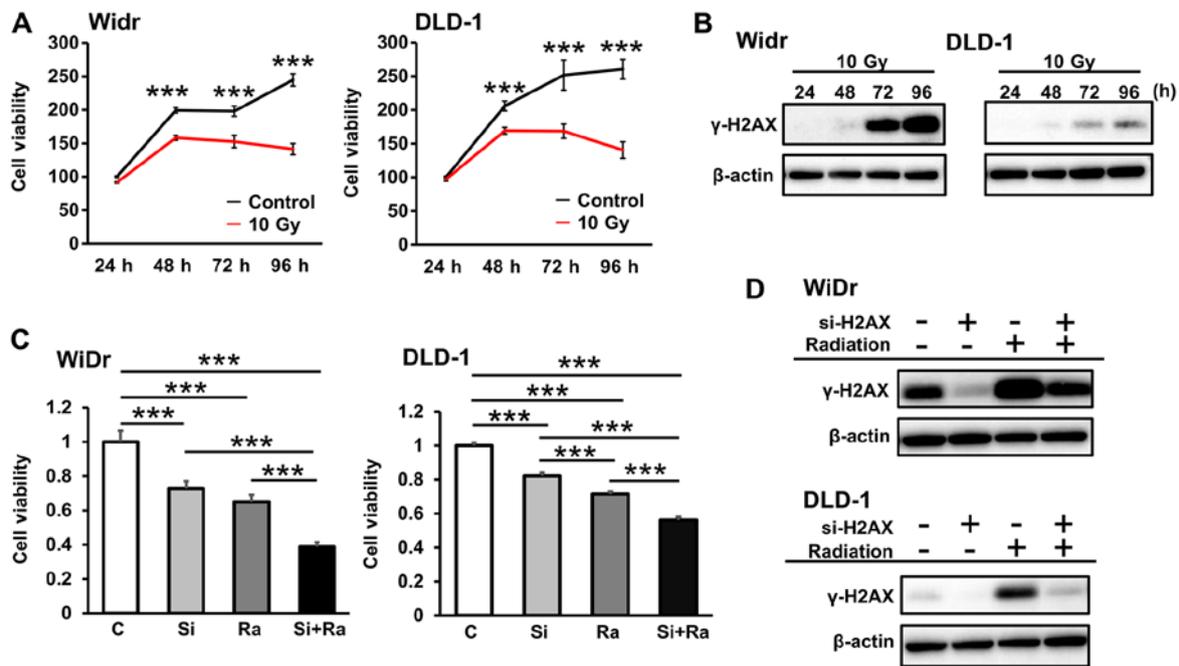


Figure 3. Association between irradiation and  $\gamma$ -H2AX expression in CRC cells. (A) Cell viabilities at 24, 48, 72 and 96 h after 10 Gy irradiation in two CRC cell lines. (B) The protein expression of  $\gamma$ -H2AX in irradiation-treated CRC cells. The experimental conditions were the same as in (A). (C) Cell viability after irradiation and siR-H2AX combination treatment in CRC cells. The effects were assessed 72 h after irradiation. C, control; Si, siR-H2AX; Ra, irradiation. (D) The protein expression levels of  $\gamma$ -H2AX after single or combination treatment. The experimental conditions were the same as in (C). \*\*\* $P < 0.001$  as indicated. H2AX, H2A histone family member X; CRC, colorectal cancer; siR or Si, small interfering RNA; C, control; Ra, irradiation.

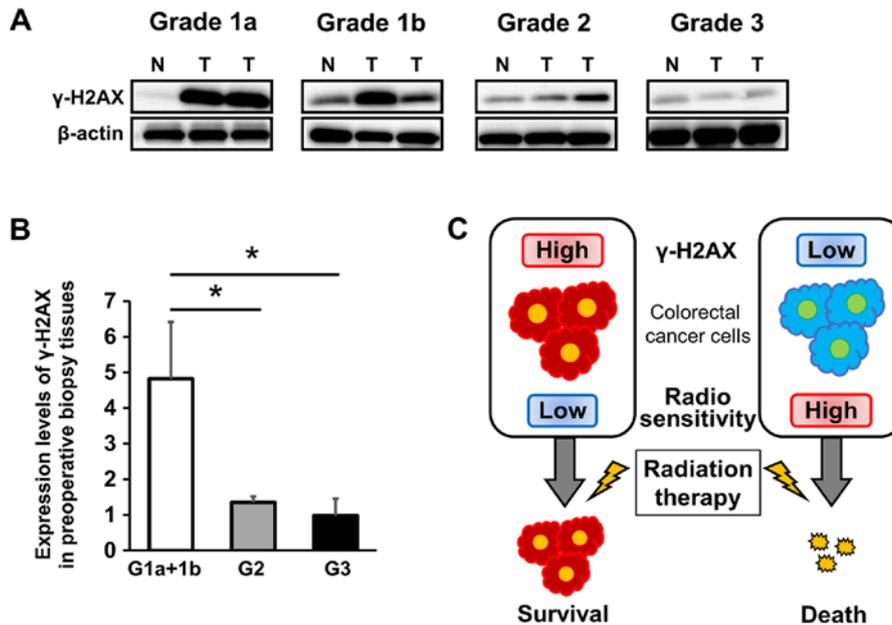


Figure 4. Association between preoperative chemoradiation therapy and  $\gamma$ -H2AX expression in clinical rectal cancer specimens. (A) Representative protein expression of  $\gamma$ -H2AX in each grade of preoperative chemoradiation therapy. (B) Quantification western blot analysis results in 11 patients with advanced rectal cancer. (C) The effectiveness of radiotherapy depends on  $\gamma$ -H2AX expression in CRC. CRC cell sensitivity to radiotherapy with low expressions of  $\gamma$ -H2AX is high. Namely, the expression of  $\gamma$ -H2AX is associated with the potential for radiotherapy resistance. \* $P < 0.05$  as indicated. H2AX, H2A histone family member X; CRC, colorectal cancer; grade 1a, minimal effect; grade 1b, mild effect; grade 2, moderate effect; grade 3, marked effect in rectal cancer patients; N, normal, T, tumor.

such as cancer of the breast, bladder, and ovary (29-31). These reports support our results, and suggest that H2AX, especially in its phosphorylated form, may be a gene which is universally associated with cancer. Importantly, the expression levels of H2AX seemed to be related to MSI. Recently, MSI has been

recognized as one of the key mechanisms of carcinogenesis due to lack of mismatch repair (MMR), and has been associated with immune checkpoint blockade therapy using pembrolizumab (32). DSB immediately phosphorylates H2AX to form  $\gamma$ -H2AX (33), and  $\gamma$ -H2AX is a known sensitive marker for

DSB (11). Hence, the results met our expectations, and the elucidation of the detailed association between the roles of H2AX (non-phosphorylated form) and the acquisition of MSI remains an important issue.

In this study, we also evaluated the potential of  $\gamma$ -H2AX to predict the effectiveness of preoperative radiotherapy. The endogenous expression levels of  $\gamma$ -H2AX in WiDr cells without any therapy was higher than that in DLD-1 cells, and the effect of  $\gamma$ -H2AX suppression on cell viability in WiDr cells was stronger than that in DLD-1 cells after irradiation. Taken together, these findings imply that the expression level of  $\gamma$ -H2AX is important for radiosensitivity, and CRC cells with an elevated expression of  $\gamma$ -H2AX possess a certain tolerance to irradiation. Various predictive factors for radiotherapy have been previously reported, such as p53, ki67, Bax, Bcl-2, cyclooxygenase-2 and CD133 (34-37). However, a bona fide predictive marker for radiotherapy has yet to be established, and further research is required to identify reliable candidates.

In addition, the underlying mechanism which associates  $\gamma$ -H2AX with cell death, after irradiation, is unknown. Several molecular mechanisms have been reported, relating to genes associated with cell death and  $\gamma$ -H2AX. It has been shown that the inhibition of caspase-4 activation interferes with  $\gamma$ -H2AX in CRC cells (38). The relationship with poly(ADP-ribose) polymerase (PARP), which is activated by DNA damage similar to H2AX, is extremely important because PARP-inhibitors are, clinically, expected to become novel anticancer drugs (39). In addition, epigenetic regulation should be considered. For example, microRNA-138 regulates DNA damage by targeting H2AX (40), and H2AX phosphorylation regulates apoptosis in lung cancer cells via the microRNA-3196/PUMA pathway (41).

The present study had some limitations. First, the number of cases used in the investigation of sensitivity to preoperative chemoradiotherapy was small (only eleven cases). To enhance the reliability of our findings, larger studies and confirmatory studies are needed. Second, we also have to consider the influence of chemotherapy on  $\gamma$ -H2AX expression. In many cases, radiotherapy is not performed alone, as a preoperative therapy for CRC. Fluorouracil and oxaliplatin, which are generally used in preoperative chemoradiotherapy, induce DNA damage. In this study, we attempted to focus on the association between H2AX and radiosensitivity, and avoided an extremely complicated experimental system. Third, regarding the experiments using CRC cells, the association of  $\gamma$ -H2AX with radiosensitivity was examined in a limited environment. Although a comparative investigation in a xenografted mouse model, using CRC cells with either high- or low-  $\gamma$ -H2AX expression, might support our findings, we selected human specimens because of concerns that the immune response would be insufficient in an animal model. Fourth, our findings of an association between H2AX and MSI were preliminary results. Considering recent advances in immune checkpoint blockade therapy, it is time to clarify the detailed mechanisms of this association.

In conclusion, the expression levels of H2AX and  $\gamma$ -H2AX were upregulated in CRC cells. Moreover, this upregulation may be associated with MSI. In radiotherapy, sensitivity was enhanced by the suppression of  $\gamma$ -H2AX, and  $\gamma$ -H2AX showed potential as a novel predictive marker of the effectiveness of preoperative radiotherapy in CRC patients.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

SK, KTas and KTani conceived and designed the current study. SK performed *in vivo* experiments, NK performed *in vitro* experiments and KK analyzed datasets. TT, SK, NK, KTas, KTani, YInom, YIm, RT, YInou, MK, KK, KU, MY, JO, KTana and SWL interpreted and analyzed the data. SK, KTani, MY, KTana and JO provided materials and funding. SK, KTas, KTani wrote and revised the manuscript. KU supervised the current study. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the research Ethics Committee of Osaka Medical College (approval no. 1280; 2nd September 2013) and was conducted in accordance with the Helsinki Declaration. Before treatment, each patient provided written informed consent regarding the use of their tissues in this research.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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