Positive immunohistochemical staining of γH2AX is associated with tumor progression in gastric cancers from radiation-exposed patients

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Abstract. To elucidate the mechanism of radiation-induced cancers, molecular analysis of cancers in atomic bomb (A-bomb) exposure is important. DNA double-strand breaks (DSBs) are thought to be caused by the deleterious effects of ionizing radiation, and γH2AX (serine 139 phosphorylated form of histone H2AX) is reported to be a significant marker for DSBs. In the present study, we performed immunohistochemical analysis of γH2AX in gastric cancers (GCs) from 66 exposed and 47 non-exposed patients who developed GC after the bombing. Of the 47 GCs from non-exposed patients, 6 (13%) cases showed nuclear positive staining for γH2AX, whereas of the 66 GCs from exposed patients, 20 (30%) cases were positive (P=0.0405). However, among stage I GC, there was no significant difference in γH2AX expression frequency between exposed patients and non-exposed patients. Among exposed patients, stage II-IV cases were more frequently positive for γH2AX than stage I cases (P=0.0197). Among GCs from non-exposed patients, γH2AX staining showed no significant association with Lauren's classification, depth of invasion, lymph node metastasis or TNM stage. These results suggest that the characteristics of tumor cells differ between GCs from exposed and non-exposed patients. DSBs may be involved in progression of GC in exposed patients.

Introduction

More than 60 years have passed since atomic bomb (A-bombs) exposure in Hiroshima and Nagasaki, Japan. A prospective cohort study (Life Span Study, LSS) of 120,000 subjects is being conducted by the Radiation Effects Research Foundation (RERF) (1). It was reported that exposure to ionizing radiation (IR) increases the risk of leukemia and other cancers (2), and damage to nuclear DNA likely represents an initiating event for carcinogenesis. Increases in cancer risk due to exposure to IR are based on epidemiologic studies of exposed human populations, mainly the A-bomb survivors of Hiroshima and Nagasaki (3). Solid cancers, including breast, colon, lung and stomach cancers, have a long latency period, and the excess relative risks (RRs) of solid cancers remain high, specifically among those exposed when young (1). Although approximately half of the LSS members are now deceased, cancer mortality in the LSS has continued to increase as this population ages, and it is anticipated to peak in 2015.

According to the World Health Organization, gastric cancer (GC) is the fourth most common malignancy worldwide, with approximately 870,000 new cases occurring yearly. Cancer develops as a result of multiple genetic and epigenetic alterations (4,5). Although several genetic alterations, including mutations in *TP53* and *BRAF*, have been reported in selected cancers of A-bomb survivors (6-8), specific mutations for radiation-associated cancers have not been reported.

DNA double-strand breaks (DSBs) are thought to be caused by the deleterious effects of IR (9,10). DSBs can induce chromosomal aberrations that cause cells to malfunction, resulting in cell death or tumorigenesis (10). One of the earliest steps in the cellular response to DSBs is the phosphorylation of histone H2AX at serine 139 (γH2AX), the site of γ-phosphorylation (11). H2AX can be phosphorylated by several phosphoinositide-3 (PI3) kinases including ataxia telangiectasia mutated (ATM), DNA-dependent protein kinase (DNA-PK) and ataxia telangiectasia and Rad3 related (ATR) (12). The number of resulting γH2AX foci has been correlated directly with the number of DSBs produced by IR (13,14). Therefore, the number of γH2AX foci is a significant marker for DSBs. Immunohistochemical analyses of γH2AX have been reported for human cancers of the urinary bladder,

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breast, lung, colon and prostate (15-17). It was also reported that γH2AX-positive cells are present in colorectal cancer (CRC) and precursor lesions, such as adenoma, but not in normal colonic epithelium (15). Furthermore, invasive CRCs were reported to show less γH2AX staining than adenomas (15). These results suggest that staining of γH2AX correlates with DNA damage checkpoint activation in premalignant lesions. Therefore, the existence of γH2AX foci might be a useful and sensitive marker of cancer, especially for detecting cancers or precursor lesions in A-bomb survivor, because IR induces DSBs. However, there are no reports of immunohistochemical analyses of γH2AX in GCs from either IR-exposed patients or -non-exposed patients. Therefore, in the present study, we performed immunohistochemical analysis of γH2AX in 113 GCs derived from A-bomb survivors.

Patients and methods

Patients and tumor specimens. For immunohistochemical analysis, we used formalin-fixed, paraffin-embedded archival tissues from 113 patients with GC who underwent surgery between 1975 and 2005 at Hiroshima University Hospital (Hiroshima, Japan) or an affiliated hospital. Only patients who did not undergo preoperative radio- or chemotherapy were enrolled in the study. All 113 patients were A-bomb survivors (LSS cohort members) in Hiroshima, Japan. Although these patients were survivors who developed GC after the bombing, they were further classified according to their level of radiation exposure (i.e., ≥5 mGy and <5 mGy were defined as 'exposed' and 'non-exposed', respectively). Our population comprised 66 exposed (median dose, 51 mGy; range, 5-2601 mGy) and 47 non-exposed patients (median dose, 0 mGy; range, 0-4 mGy).

Tumor staging was performed according to the Union Internationale Contre le Cancer (UICC) system (18). Histologic classification was carried out according to the Lauren classification system (19). The detailed procedures for acquiring informed consent from study patients and collecting tissue specimens were described previously (20). In accordance with the Ethical Guidelines For Human Genome/Gene Research enacted by the Japanese Government, tissue specimens were collected and used after approval from the Ethical Review Committee of the Hiroshima University School of Medicine and from the ethical review committees of collaborating organizations.

Radiation dose. A-bomb radiation doses were estimated with the DS02 system (21).

Immunohistochemistry. From each patient, one representative tumor block, including the tumor center and invasive front as well as tumor-associated non-neoplastic mucosa, was examined by immunohistochemistry. In cases of large, late-stage tumors, different sections were examined to include representative areas of the tumor center as well as of the lateral and deep tumor invasive fronts.

Immunohistochemical detection of γH2AX was performed with a mouse monoclonal antibody (Upstate Biotechnology, Chicago, IL, USA) and Dako Envision Kit (Dako, Carpinteria, CA). In brief, sections were pretreated by microwaving (500 W) in citrate buffer (pH 6.0) for 15 min to retrieve antigenicity. After endogenous peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, sections were incubated with normal goat serum (Dako) for 20 min to block non-specific antibody binding sites. Sections were then incubated with anti-γH2AX (diluted 1:200) for 1 h at room temperature followed by incubation with peroxidase-labelled anti-mouse IgG for 60 min. Staining was completed with a 10-min incubation with the substrate-chromogen solution. Sections were counterstained with 0.1% hematoxylin. Appropriate negative controls were created by omission of the primary antibody. All slices were evaluated without knowledge of the clinical data.

Double immunofluorescence staining. Double immunofluorescence staining for dewaxed sections was performed with mouse monoclonal anti-γH2AX antibody (Upstate) with rabbit polyclonal anti-H2AX antibody (Upstate) or mouse monoclonal anti-γH2AX antibody with a rabbit polyclonal antibody against the activated form of caspase-3 (Promega, Madison, WI, USA). Microwave pretreatment in citrate buffer was performed for 15 min to retrieve antigenicity. Sections were then incubated with normal goat serum for 30 min to block non-specific antibody binding sites. Sections were treated consecutively at room temperature with primary antibodies for 60 min, and immunocomplexes were detected with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 546-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA).

Statistical methods. Associations between clinicopathologic variables and immunostaining for γH2AX were analyzed by Fisher's exact test. A P-value <0.05 was considered statistically significant.

Results

Of 113 GC from A-bomb survivors, 48 (42%) showed nuclear staining of γH2AX (Fig. 1A). These 48 cases comprised 26 GC cases with diffuse staining for γH2AX and 22 GC cases with staining of γH2AX only in superficial portions (Fig. 1B) or in necrotic debris in the lumen (Fig. 1C). We confirmed that γH2AX yielded granular, nuclear staining (Fig. 1D). H2AX showed ubiquitous staining in GC (Fig. 1E). It was reported previously that γH2AX is expressed during early apoptosis triggered through the caspase-3/caspase-activated DNase (CAD) pathway (22,23). Double immunofluorescence staining revealed that γH2AX-positive tumor cells in superficial portions or necrotic debris were also positive for the activated form of caspase-3 (a marker of apoptosis) (Fig. 1F). Because we believed that γH2AX staining induced by apoptosis was not related to IR, cases with superficial staining and staining of necrotic debris were excluded from the positive cases. In contrast, the percentage of γH2AX-stained tumor cells was >5% in 26 GC cases showing diffuse staining; we considered these as positive cases. Twenty-four of 26 GC cases had from 5% to 10% of γH2AX-stained tumor cells. In particular, remaining two cases had >30% of γH2AX-stained tumor cells, both of which were α-fetoprotein (AFP)-positive GC.

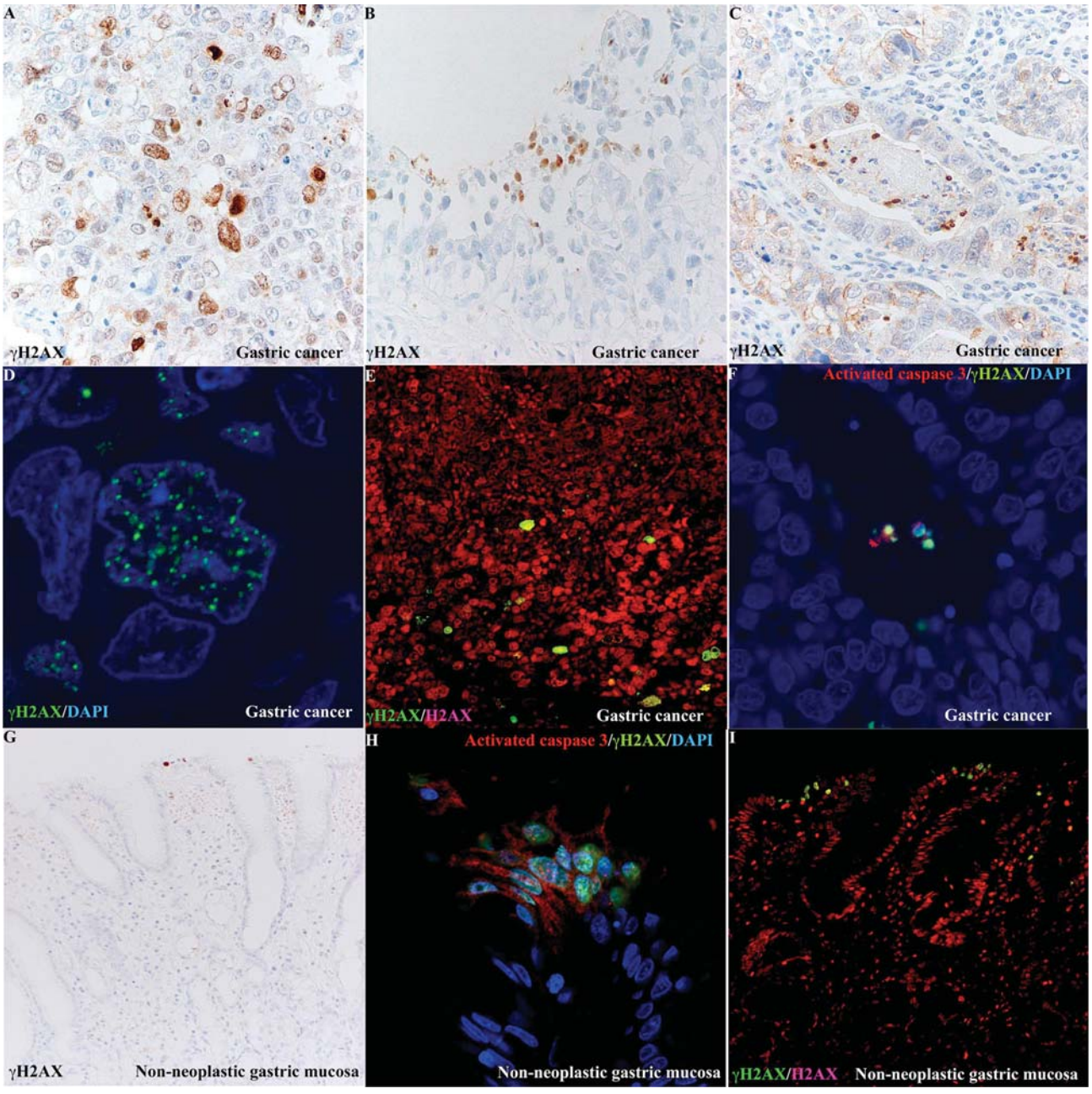

Figure 1. Immunohistochemical analysis of γH2AX in gastric carcinoma (GC) tissues (A-F) and non-neoplastic gastric mucosa (G-I). Staining for γH2AX in GC. Schematic representation of a positive case (A). Tumor cells or necrotic debris with γH2AX staining are located at the superficial portion of GCs (B) or in the lumen of the tumor gland (C). Granular staining of γH2AX is found in the nucleus (D). Staining for H2AX. H2AX shows ubiquitous staining both in GC (E) and non-neoplastic gastric mucosa (I). Some surface cells in non-neoplastic gastric mucosa also express γH2AX (G). Double immunofluorescence staining of the activated form of caspase-3 and γH2AX. Necrotic debris in the lumen of GCs (F) and superficial apoptotic cells in non-neoplastic gastric mucosa (H) are shown. Cells were imaged with a fluorescence microscope as described in Materials and methods.

We analyzed the association between γH2AX staining and clinicopathologic parameters in GCs from 66 IR exposed and 47 non-exposed patients (Table I). When all tumor stages were considered, γH2AX staining was detected in 20 (30%) of 66 exposed patients and 6 (13%) of 47 non-exposed patients (P=0.0405). Because IR is a carcinogen and can increase an individual's risk of tumor development, we analyzed immunohistochemical staining for γH2AX in early-stage GCs. In GCs showing T1 (tumor invades lamina propria or submucosa), N0 (no regional lymph node metastasis), or stage I, γH2AX positivity did not differ significantly between exposed and non-exposed patients. In contrast, among T2-4 GCs, γH2AX was expressed more often in exposed patients than in non-exposed patients (P=0.0236). In stage II-IV GCs, γH2AX expression showed a marginally significant difference between exposed patients and non-exposed patients (P=0.0602).

Of the 66 GCs from exposed patients, γH2AX was present in 20 (30%). γH2AX expression in GCs from exposed patients was associated significantly with depth of invasion (P=0.003) (Table II). Furthermore, γH2AX staining was observed more frequently in stage II-IV GCs than in stage I GCs (P=0.0197) (Table II). In contrast, the presence of γH2AX in GCs from non-exposed patients showed no signi-

Table I. Expression of γH2AX in GC and its association with clinicopathologic variables.

	No. of cases	γH2AX positive (%)	P-value
GC	113	26 (23)	
Exposed patients	66	20 (30)	
Non-exposed patients	47	6 (13)	0.0405
Intestinal type[a]			
Exposed patients	34	9 (26)	
Non-exposed patients	33	5 (15)	0.3689
Diffuse type[a]			
Exposed patients	32	11 (34)	
Non-exposed patients	14	1 (7)	0.073
Depth of invasion[b]			
T1			
Exposed patients	15	0 (0)	
Non-exposed patients	19	2 (11)	0.492
T2-4			
Exposed patients	51	20 (39)	
Non-exposed patients	28	4 (14)	0.0236
Lymph node metastasis[b]			
N0			
Exposed patients	25	5 (20)	
Non-exposed patients	24	2 (8)	0.4174
N1-4			
Exposed patients	41	15 (37)	
Non-exposed patients	23	4 (17)	0.1552
TNM stage[b]			
I			
Exposed patients	21	2 (10)	
Non-exposed patients	23	2 (9)	1.000
II-IV			
Exposed patients	45	18 (40)	
Non-exposed patients	24	4 (17)	0.0602

GC, gastric carcinoma. [a]Histologic classification of GC was according to the Lauren classification system. [b]Tumor stage was according to the Tumor-Node-Metastasis (TNM) staging system.

ficant correlation with Lauren's classification, depth of invasion, lymph node metastasis or TNM stage (Table II). There was no significant association between γH2AX staining and radiation dose at the time of A-bombing (data not shown).

In non-neoplastic gastric mucosa or intestinal metaplasia adjacent to the tumor, only a few superficial cells in both exposed and non-exposed patients showed immunostaining of γH2AX (Fig. 1G) and activated form of caspase-3 (Fig. 1H). H2AX showed ubiquitous immunostaining (Fig. 1I).

Discussion

While the DNA damage response plays a major role in tumor suppression, how this response contributes to suppression of stomach tumorigenesis remains unclear. DSBs of chromosomal DNA are thought to be caused by the hazardous effects of IR and may result in chromosomal translocations, deletions or loss of genetic information, which are all causatively linked to tumorigenesis (15). Therefore, DSBs may be involved in radiation-associated gastric carcinogenesis among A-bomb survivors. In the present study, we provide immunohistochemical evidence that γH2AX is expressed in 13% of GCs from non-exposed patients and 30% of GCs from exposed patients. Because IR is a carcinogen and can increase an individual's risk of tumor development, DSBs appear to play a more important role in early-stage GC rather than late-stage GC. In fact, γH2AX is reported to be expressed commonly in early precursor lesions in urinary bladder, breast, lung, colon and prostate (15,17). However, in

Table II. Immunohistochemical analysis of γH2AX in GCs from exposed and non-exposed patients.

	No. of cases	γH2AX positive (%)	P-value
Exposed GC patients	66	20 (30)	
Histology[a]			
Intestinal type	34	9 (26)	
Diffuse type	32	11 (34)	0.5946
Depth of invasion[b]			
T1	15	0 (0)	
T2-4	51	20 (39)	0.003
Lymph node metastasis[b]			
N0	25	5 (20)	
N1-4	41	15 (37)	0.1792
TNM stage[b]			
I	21	2 (10)	
II-IV	45	18 (40)	0.0197
Non-exposed GC patients	47	6 (13)	
Histology[a]			
Intestinal type	33	5 (15)	
Diffuse type	14	1 (7)	0.6532
Depth of invasion[b]			
T1	19	2 (11)	
T2-4	28	4 (14)	1.000
Lymph node metastasis[b]			
N0	24	2 (8)	
N1-4	23	4 (17)	0.4158
TNM stage[b]			
I	23	2 (9)	
II-IV	24	4 (17)	0.6662

GC, gastric carcinoma. [a]Histologic classification of GC was according to the Lauren classification system. [b]Tumor stage was done according to be the Tumor-Node-Metastasis (TNM) staging system.

the present study, there was no significant difference in γH2AX staining between stage I GCs from exposed patients and non-exposed patients. Furthermore, in intestinal metaplasia adjacent to the tumor, which is considered to be a gastric precancerous lesion, staining of γH2AX was not observed in epithelial or stromal cells. These results suggest that DSBs are less likely to be involved in the genesis of GCs.

In contrast, in exposed patients, γH2AX-positive GC cases showed more advanced depth of invasion and higher TNM stage than γH2AX-negative GC cases, suggesting that DSBs may participate in progression of GC in exposed patients. It has been reported that deregulated c-myc expression induces DNA damage and the formation of γH2AX (24). However, mammalian SWI/SNF complexes facilitate DSBs repair by promoting γH2AX product (25), and we reported previously that increased expression of BRG1, a component of the SWI/SNF complex, is associated with advanced-stage GCs (26). It is possible that such signals may also contribute to γH2AX activation in GCs from exposed patients. Taken together, the molecular mechanisms that underlie phosphorylation of γH2AX may differ between IR-exposed patients and -non-exposed patients. It is also possible that because a single DSB can result in chromosomal translocations, deletions or loss of genetic information, several genes associated with tumor progression may be deleted in γH2AX-positive GC cases. Further studies are needed to identify these mechanisms.

In conclusion, DSBs do not appear to be characteristic alterations in stomach carcinogenesis in IR-exposed patients. However, immunohistochemical staining of γH2AX is increased with tumor progression in GCs from exposed patients. Although it is unclear whether all GCs from exposed patients in the present study were radiation-induced cancers, DSBs may serve as a marker for progression of GCs.

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