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,
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 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% H2O2-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.
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-
significant 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 commonly in early precursor lesions in urinary bladder, breast, lung, colon and prostate (15,17). However, in

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<tr>
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<td>47</td>
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<tr>
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<td>20 (30)</td>
<td>6 (13)</td>
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<td>33</td>
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<th>T2-4</th>
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<tr>
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<td>γH2AX positive (%)</td>
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<td>20 (39)</td>
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<tr>
<td>Non-exposed patients</td>
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<td>23</td>
</tr>
<tr>
<td>γH2AX positive (%)</td>
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</tr>
<tr>
<td>Non-exposed patients</td>
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<td>24</td>
</tr>
<tr>
<td>γH2AX positive (%)</td>
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<td>18 (40)</td>
</tr>
<tr>
<td>P-value</td>
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<td>0.0602</td>
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GC, gastric carcinoma. *Histologic classification of GC was according to the Lauren classification system. *Tumor stage was according to the Tumor-Node-Metastasis (TNM) staging system.
In the present study, there was no significant difference in \( \gamma \)H2AX 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 \( \gamma \)H2AX 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, \( \gamma \)H2AX-positive GC cases showed more advanced depth of invasion and higher TNM stage than \( \gamma \)H2AX-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 \( \gamma \)H2AX (24). However, mammalian SWI/SNF complexes facilitate DSBs repair by promoting \( \gamma \)H2AX 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 \( \gamma \)H2AX activation in GCs from exposed patients. Taken together, the molecular mechanisms that underlie phosphorylation of \( \gamma \)H2AX 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 \( \gamma \)H2AX-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 \( \gamma \)H2AX 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.

Table II. Immunohistochemical analysis of \( \gamma \)H2AX in GCs from exposed and non-exposed patients.

<table>
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<tr>
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<th>No. of cases</th>
<th>( \gamma )H2AX positive (%)</th>
<th>P-value</th>
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<tr>
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<tr>
<td>Histology(^a)</td>
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<tr>
<td>Intestinal type</td>
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<td>9 (26)</td>
<td></td>
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<tr>
<td>Diffuse type</td>
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<td>11 (34)</td>
<td>0.5946</td>
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<tr>
<td>Depth of invasion(^b)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>15</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>T2-4</td>
<td>51</td>
<td>20 (39)</td>
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<td>Lymph node metastasis(^b)</td>
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<tr>
<td>I</td>
<td>21</td>
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<tr>
<td>II-IV</td>
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<td>47</td>
<td>6 (13)</td>
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<td>4 (17)</td>
<td>0.6662</td>
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GC, gastric carcinoma. \(^a\)Histologic classification of GC was according to the Lauren classification system. \(^b\)Tumor stage was done according to the Tumor-Node-Metastasis (TNM) staging system.
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