Sixty years of follow-up of Hiroshima and Nagasaki survivors:
Current progress in molecular epidemiology studies

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Received 23 July 2007; received in revised form 30 January 2008; accepted 4 February 2008
Available online 12 February 2008

Abstract

This article provides an overview of the on-going molecular epidemiology studies among atomic-bomb survivors conducted at the Radiation Effects Research Foundation in Japan. The focus is on: (a) inter-individual variations in sensitivity to radiation-induced somatic mutations (glycophorin A (GPA) mutations) and their potential relevance to differences in susceptibility to radiation-related cancers and (b) the role of specific mutations/rearrangements in radiation-induced thyroid and colorectal cancers. The glycophorin A mutant fractions showed large differences between the survivors at each of the estimated bone marrow doses. Of note is the finding at doses >1 Gy; that the slope of the mutant fraction was significantly higher in the ‘cancer group’ than in the ‘non-cancer group’. This study provided the basis for validating the use of γH2AX and reticulocyte micronucleus assays for evaluating radiosensitivity differences and genetic instability, respectively, in our studies in the coming years. Preliminary results from our molecular oncology studies on adult-onset papillary thyroid cancer provide evidence for the induction of RET/PTC rearrangements and BRAF point mutation (both known to be early stage events in adult-onset papillary thyroid cancer) but with a difference: cases associated with the rearrangements were more frequent at high doses, and developed sooner than those with BRAF mutation. In the case of colorectal cancer, the results suggest that radiation exposure might influence microsatellite instability (MSI) status through MSI-related epigenetic and genetic alterations—processes that might occur in the early stage of colorectal carcinogenesis.

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Keywords: Radiation; Atomic-bomb survivors; Somatic mutation; Oxidative stress; Colorectal carcinogenesis; Microsatellite instability; Thyroid carcinogenesis

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doi:10.1016/j.mrrev.2008.02.001
1. Introduction

Molecular epidemiology studies among atomic-bomb survivors (A-bomb survivors) seek to deepen our understanding of the mechanisms by which ionizing radiation affects human health. Their importance will be evident in future prevention of radiation-associated diseases: not only confined to establishing safeguards against radiation exposure, but also in developing the ways and means to identify high-risk individuals and more efficient approaches to prevent radiation-associated diseases. The mechanistic study group in our laboratory comprises two subgroups and the following research themes: radiation effects on immune functions (specifically, T-cell mediated immunity) and aging-associated persistent inflammation [1], a genome approach relative to individual risks of radiation-associated diseases, somatic mutability in relation to cancer occurrence and molecular oncology analyses of radiation-associated cancers. These studies are based on analyses of invaluable biological resources obtained from the two cohort studies conducted at the Radiation Effects Research Foundation (RERF); the Life Span Study (LSS) with 120,000 survivors, and the Adult Health Study (AHS) with 20,000 survivors, which has conducted biennial medical examinations among a subcohort of the LSS.

In this article, we report on current progress in selected research themes of our laboratory relating to two critical issues: (1) inter-individual variations in sensitivity to radiation-induced somatic mutation and (2) relationship between radiation exposure and various gene alterations in carcinogenesis. The first issue was addressed using biomarkers of individual radiation sensitivity and presumed radiation-induced genetic instability, among A-bomb survivors, based on our previous findings in the glycoporphin A (GPA) mutant fraction (Mf) study, as well as biomarkers of inflammation and production of reactive oxygen species (ROS). In this GPA Mf study, we demonstrated large inter-individual variation in the GPA Mf response of survivors exposed to atomic radiation, as well as a significantly higher sloped GPA Mf dose–response curve for doses ≥ 1 Gy, when the cancer group was compared with the non-cancer group [2]. This study prompted two questions: (1) are individual sensitivities to radiation-induced genetic damage responsible for the inter-individual variation noted in dose-dependent GPA Mf response? And (2) is radiation-induced genetic instability involved in the dose-dependent increase of GPA Mf? To address these questions, we considered several biomarkers and assessed and verified the suitability of two of them, namely the γH2AX and reticulocyte micronucleus endpoints. Both of these biomarkers appear applicable to evaluation of individual sensitivity to radiation-induced genetic damage and instability [3,4]. In parallel with the somatic mutation study, we have observed dose-dependent increase of various inflammatory biomarkers among A-bomb survivors [5]. This enhanced and persistent inflammation needs to be examined in relation to the endogenous production of reactive oxygen species (ROS), linking inflammation and somatic mutations.

The second issue can be further broken down into two questions: which types of genetic alterations e.g., chromosome aberration or point mutation, preferentially occurred in radiation-associated cancer, and whether or not radiation influenced epigenetic alterations during carcinogenesis. Adult-onset papillary thyroid cancer seems to be a good model for examining and contrasting chromosome aberrations and point mutations, since the major initial event of this cancer is either RET/PTC rearrangements or BRAF point mutation, which appear to occur in a mutually exclusive manner. It is well recognized that colorectal cancer can be phenotyped according to microsatellite stability. Accordingly, we have begun to analyze microsatellite instability (MSI), along with methylation status of MLH1, using cancer tissue specimens from A-bomb survivors. Although these studies are still preliminary and continuing, we report here our interim results [6].

2. Radiation-induced genetic damage and inflammation

2.1. GPA Mf study in the AHS cohort

We have conducted a prospective study among a total of 1723 MN heterozygous AHS participants who were cancer free and without cancer history at the time (1988–1996) of GPA Mf measurements, in which mutated erythrocytes, namely hemi-zygous Mφ or Nφ cells, were counted by flow cytometry [2]. During a follow-up period that lasted until 2000, a total of 186 cancer cases were identified. The major findings from this study were: (1) a radiation dose-dependent increase of GPA Mf was observed in the total population, as well as in the cancer and cancer-free participants in Hiroshima and Nagasaki, and (2) the slope of the GPA Mf dose-response above 1 Gy was significantly higher in the Hiroshima cancer group than in the cancer-free group within the same high dose region (Fig. 1). These findings imply that inter-individual variations in GPA Mf might indicate individual differences in somatic mutability response to radiation exposure, and that individuals with higher mutability in GPA Mf response could have increased risk of radiation-associated cancer. It also suggests that the inter-individual variation in GPA Mf might involve differences in hematopoietic stem cell repair capacity of DNA double-strand breaks induced by high-dose irradiation. Alternatively, differences might exist between individuals in terms of persistent radiation-induced genetic instability within the hematopoietic system.

Another important message of Fig. 1 is that the wide variation in GPA Mf among individuals exposed to high radiation doses was not merely the result of random errors in measurement, or in dose dosimetry. It was also thought to primarily reflect inter-individual differences in sensitivity to radiation: higher responders to given radiation doses had higher probability of developing radiation-associated cancer. Of course, unidentified factors other than radiation sensitivity are possibly involved in this inter-individual variation, specifically considering the long period elapsed since atomic radiation exposure and other environmental factors influencing GPA Mf [7]. The large reduction in size of the stem-cell pool as a consequence of cell destruction by A-bomb irradiation might cause stochastic fluctuation of GPA Mf derived from a limited
number of mutant stem cells [8]. Therefore, a new study will be required with use of biomarkers more specific to acute and delayed effects of radiation.

These results provided the basis to ask two further questions. One question is whether or not individual sensitivity to radiation-induced genetic damage was reflected by the noted inter-individual variation in GPA Mf as a function of radiation dose. The other is whether genetic instability was induced and contributed to the noted dose-dependent increase of GPA Mf.

To answer these questions, we recently developed a gH2AX assay system to evaluate individual sensitivity to the immediate effects of set doses of radiation, as well as a reticulocyte micronucleus assay system to investigate delayed effects of radiation, namely genetic instability.

2.2. Biomarkers for radiation sensitivity and genetic instability

Various biomarkers measuring radiation-induced cellular damage, such as DNA strand-breaks, chromosomal damage and lethality, have been studied to assess individual sensitivity to radiation exposure in vitro. However, these bioassays require considerable labor and technical skill to achieve reliable and reproducible results. Thus, assay platforms that can more easily and reliably measure radiation-induced cellular damage need to be identified, especially to facilitate biomarker assays and associated risk estimation in a large study population. For this purpose, we thought that flow cytometry was the most applicable method because of its performance characteristics: i.e., high throughput and high-sensitivity.

It is known that histone H2AX, a subfamily of histone H2A, is phosphorylated and forms foci (γH2AX foci) at the sites of DNA double-strand breaks induced by ionizing radiation [9]. The number of γH2AX foci closely corresponds to that of DNA double-strand breaks in cells [10], and counting γH2AX foci has frequently been used as a more sensitive DNA damage marker than more conventional assays, such as pulse-field gel electrophoresis, neutral single cell electrophoresis (Comet assay), or DNA elution assay [11]. We recently have established a flow cytometry system for detecting γH2AX induced by in vitro irradiation, using cultured T lymphocytes, which are readily available from healthy individuals, and attempted to validate its suitability for analysis of individual radiosensitivities in human populations [3]. Because γH2AX focus formation appears not only in genomically damaged cells, but also in cells undergoing DNA synthesis and mitosis [12,13], improved assays to detect level of radiation-induced γH2AX foci were required. Toward this end, we simultaneously analyzed γH2AX expression and DNA content, and γH2AX levels were determined in cell fractions accurately gated for G0/G1 phase cells. Such cell-cycle-specific analysis is feasible for flow cytometry, but not for conventional fluorescence microscopy. Short-term (7 days) cultured T lymphocytes, but not uncultured, freshly isolated lymphocytes from peripheral blood, exhibited significant inter-individual differences in level of γH2AX induced by in vitro X-irradiation (Fig. 2). The reason why no obvious inter-individual differences were detected in the assay using uncultured lymphocytes remains elusive, but substantial differences in metabolic status, such as differences in the levels of radical scavenger proteins, between cultured and uncultured lymphocytes might result in differences in radiation sensitivity (discussed in reference [3]). Our assay system also provides good reproducibility, as well as a capacity to detect significant inter-individual differences between the responses of T lymphocytes from six healthy donors 6 h after 4 Gy of X-irradiation [3]. Variation in the level of γH2AX in cultured T lymphocytes of these individuals was about 1.5-fold. Differences in lymphocyte subsets either before or after culture were not responsible for this noted variance in individual radiosensitivity. Thus, our γH2AX assay system using cultured T lymphocytes appears to be useful for the rapid and reliable assessment of individual radiation sensitivity. We have already
confirmed that T lymphocytes cultured from cryopreserved lymphocytes are also useful for this radiation sensitivity assay. Evaluation of radiation sensitivity using cryopreserved lymphocytes from A-bomb survivors for whom GPA MF has been analyzed is underway.

It has been recently reported that chromosomal alterations are indicated not only in cells that have been directly irradiated, but also in unirradiated neighboring cells, or descendants of irradiated cells [14]. Such delayed radiation effects, termed radiation-induced genetic instability effects, are thought to represent adverse cellular consequences, such as linked bystander and occasionally malignant transformation [14]. Although radiation-induced genetic instability is well characterized in vitro, evidence from in vivo studies has been limited due to lack of reliable bioassays capable of detecting and distinguishing “delayed type” chromosomal alterations induced by direct radiation effects versus those induced indirectly. Thus, to ensure identification of both delayed and direct radiation effects in vivo, we applied a flow cytometry-based reticulocyte micronucleus assay [15–17]. Because micronuclei in reticulocytes are chromosomal segments separated from entire chromosomes during the enucleation stage of erythrocyte maturation, and because the estimated lifespan of reticulocytes in vivo is as short as a few days [18], reticulocyte micronuclei observed more than 1 month after irradiation are not those that have been directly induced by radiation but those that have arisen in the course of normal erythropoiesis (Fig. 3). By utilizing this post-irradiation time discrepancy in the appearance of reticulocyte micronuclei, we were able to analyze delayed radiation effects separately from direct effects in whole-body X-irradiated mice [4]. In irradiated mice, we detected an acute effect from radiation dose as small as 0.1 Gy 2 days after irradiation, and a significant difference in the radiation-dose response between BALB/c and C57BL/6 mice (regression $P < 0.001$). As for delayed radiation effects, we also observed significantly increased frequencies of reticulocyte micronuclei in both BALB/c and C57BL/6 mice (1.6- and 1.3-fold increases compared with age-matched controls, Mann–Whitney, $P = 0.035$ and 0.039, respectively), 1 year after irradiation with 2.5 Gy of X-rays. Interestingly, there was also a significant mouse strain difference in the delayed radiation effect (Mann–Whitney, $P = 0.028$). It was therefore concluded that delayed effects of radiation on the murine hematopoietic system can persist in vivo for prolonged periods and that there were differences by mouse strain in susceptibility to such delayed radiation effects.

Two possibilities that are not mutually exclusive have been proposed for radiation-induced genetic instability [14]. One possibility is that cells not directly irradiated, but descended from irradiated cells, exhibit genetic instability. The other is that mediators, such as ROS and inflammatory cytokines, are released from irradiated cells and enhance DNA damage in unirradiated but neighboring cells to the irradiated cells (bystander effect). Our previous study indicated that low-grade inflammation may persist for more than 50 years after irradiation in A-bomb survivor populations [5]. It is intriguing to speculate that the reticulocyte micronucleus response, an
indication of genetic instability, is associated with the noted elevation of radiation-induced GPA MI and with inflammatory status and ROS production in the survivors. Several flow cytometry methods measuring reticulocyte micronuclei in human blood samples have been developed [19,20]. Application of any one of these methods would allow us to further pursue molecular epidemiological investigations using this biomarker of genomic instability among the AHS population.

2.3. Biomarkers for inflammation and reactive oxygen species

Our immunology group has previously found that A-bomb radiation exposure enhanced persistent inflammation that is often accompanied by normal physiologic aging [5]. Further, inflammation-associated production of excessive levels of free radicals (e.g., ROS) is highly mutagenic. Therefore, endogenous ROS might be a factor to be analyzed with the aforementioned biomarkers. We have recently developed a total ROS assay system to determine the total amount of oxygen-centered free radicals derived from various ROS metabolites in a blood sample [21]. Our preliminary study showed that plasma ROS levels in AHS participants significantly increased with increased radiation dose (regression $P < 0.001$), even after adjustment was made for gender, age, smoking status and body mass (unpublished data).

3. Molecular oncology analyses of radiation-associated cancers

3.1. Adult-onset papillary thyroid cancer in the LSS cohort

Epidemiological studies on the LSS cohort of A-bomb survivors have identified a significant radiation-associated solid cancer risk [22]. However, the level of risk differed from organ to organ [22]. The excess relative risk of papillary thyroid cancer in survivors was 1.2 per Sv, and it increased with radiation dose [22]. A histopathological study has revealed that the thyroid cancer found in A-bomb survivors was largely conventional papillary in nature. This is also the case of spontaneous thyroid cancer in the Japanese at large. Solid variant papillary thyroid cancer was not found in A-bomb survivors, although this cancer has been frequently observed among post-Chernobyl children.

A major early event in papillary thyroid carcinogenesis is the constitutive activation of the MAP kinase signaling pathway caused by a single genetic alteration. This alteration has been identified as one among several possible, mutually exclusive, rearrangements of the RET and NTRK-1 genes, and point mutations in the RAS and BRAF genes [23–25]. Among post-Chernobyl childhood thyroid cancers, the prevalent RET/PTC3 rearrangement was closely associated with a short latency period after exposure and also with solid variant-type of the disease [26,27]. A low prevalence of BRAF mutation has been observed in childhood papillary thyroid cancer regardless of the presence or absence of past radiation exposure [28]. On the other hand, prevalence of RET/PTC rearrangements in radiation-associated adult-onset thyroid cancer, including radiation-therapy cases, was controversial [29,30]; BRAF point mutation (BRAF$^{V600E}$) was found at high frequency in papillary thyroid cancer among adult patients without history of radiation exposure [28]. In this regard, our previous experiments in vivo and in vitro showed that X-irradiation induced RET/PTC1 rearrangement in scid mice within human thyroid tissue transplants, as well as in human thyroid cells cultured in vitro [31].

A logical first step for clarifying the mechanistic relationship between radiation exposure and the development of papillary thyroid cancer would be identification of the gene alterations that
preferentially occur during adult-onset radiation-associated thyroid carcinogenesis. Toward this end, we analyzed RET/PTC rearrangements and \( \text{BRAF}^{\text{V600E}} \) point mutation among 50 adult-onset papillary thyroid cancer cases exposed to A-bomb radiation. Relationships to radiation dose, as well as years elapsed since A-bomb radiation exposure, were evaluated. When dividing the subjects into three groups harboring \( \text{BRAF}^{\text{V600E}} \) mutation, RET/PTC rearrangements, and other unknown gene alterations, radiation dose (tertiles) responses of these groups differed: \( \text{BRAF}^{\text{V600E}} \) mutation frequency significantly decreased in groups with increased radiation dose (Cochran-Armitage \( P_{\text{trend}} < 0.001 \)) [6], while RET/PTC rearrangements showed significantly increased frequency with radiation dose (Cochran-Armitage \( P_{\text{trend}} = 0.002 \)). Furthermore, other unknown gene alterations tended to be more frequent with increased radiation dose, suggesting that radiation-associated gene alterations (possible chromosomal rearrangements) other than RET/PTC rearrangements might be involved in the adult-onset thyroid cancers of A-bomb survivors who were exposed to high radiation doses. These findings also correspond with another significant observation in the same subjects: namely that the subjects with RET/PTC rearrangements developed cancer sooner following exposure than did the subjects with \( \text{BRAF}^{\text{V600E}} \) mutation, as illustrated in Fig. 4 (Mann–Whitney, \( P = 0.029 \)). Together, the interim results obtained thus far suggest an important role of RET/PTC rearrangements in adult-onset radiation-associated thyroid carcinogenesis.

First, we determined MSI status in 24 colon and 11 rectal cancers in A-bomb survivors with defined radiation doses, in terms of six microsatellite markers. Five MSI-H cases were found among them, all in the proximal colon and none in the rectum. The median radiation dose of MSI-H colorectal cancer patients was significantly higher than that of microsatellite stable (MSS) and low-MSI (MSI-L) cancer patients (Mann–Whitney, \( P = 0.042 \)). These observations suggest a possible link between radiation exposure and MSI.

Most MSI-H sporadic colorectal cancers showed loss of expression of the MLH1 DNA repair enzyme and the methylation of its promoter. The latter is probably a major cause for inactivation of this gene [33]. We examined as well the methylation of the CpG dinucleotides within the proximal region (−231 to −228) of the MLH1 gene, since this CpG island is responsible for decreased mRNA and/or protein expression of the gene [34–36]. Using combined bisulfite restriction analysis [37], methylation of the MLH1 gene was found in five patients, whose median radiation dose tend to be higher than that of subjects with MLH1-unmethylated genes; this finding, however, is of marginal significance (Mann–Whitney, \( P = 0.06 \)). Methylation of the MLH1 gene was significantly associated with MSI status in this study (\( \chi^2 \), \( P = 0.017 \)), as was the case in other studies; three patients showed both methylation of the MLH1 gene and the MSI-H phenotype.

In addition to DNA methylation, we examined loss-of-heterozygosity (LOH) of the MLH1 gene, which is also responsible for deficient expression of the MLH1 gene [33]. We found that all five MSI-H cases carried LOH at the gene loci. Our preliminary findings imply that MSI colorectal carcinogenesis among A-bomb survivors might involve both epigenetic and genetic alterations of the MLH1 gene.

During the past decade, it has become clear that there is a “serrated polyp pathway” associated with MSI [38]. This pathway is initiated by hyperplastic polyp formation, followed by serrated adenoma, and ultimately leading to invasive cancer. Loss of the MLH1 protein followed by acquisition of MSI occurs at the late stage [39], while point mutation of the \( \text{BRAF} \) gene (\( \text{BRAF}^{\text{V600E}} \)) is recognized as an early event [40]. We therefore examined \( \text{BRAF}^{\text{V600E}} \) and found that four cases possessed this mutation. The median radiation dose of

![Fig. 4. A scheme of gene alteration types in adult-onset papillary thyroid cancer among A-bomb survivors varying with radiation dose and year elapsed since atomic radiation exposure.](Image 162x585 to 425x729)
BRAF-mutated cases was significantly higher than that of non-mutated cases.

Our results to date suggest that radiation exposure might influence MSI status through MSI-related epigenetic and genetic alterations—processes that may have occurred in the early stage of colorectal carcinogenesis. Further analysis with an increased number of cases is clearly required, however.

4. Future directions

Epidemiological follow-up study among A-bomb survivors for over half a century has provided invaluable knowledge about how atomic-radiation influenced disease outcome, and it will continue to do so in the years ahead. However, relatively little is known about the mechanisms of adverse health effects of ionizing radiation that arise late in life. For example, risk of various solid cancers increased with radiation dose and remain high, despite six decades since the exposure event. We still do not know why some organs are more sensitive to radiation exposure than others, and why cancer incidence within such organs remains high. Apart from determining risk for the population at large, a more elusive question is how to evaluate individual sensitivity to various radiation effects and how to handle risk estimation of individuals. The molecular epidemiology study at RERF seeks to find the answers to these important questions relating to the health effects of radiation.

It may be noted that the primary aim of molecular epidemiology is not necessarily to find association between radiation and disease outcome, but to assess the various radiation effects on cells, tissues, organs, and vital physiological systems and processes of the body, such as immunity and DNA repair, in terms of various biomarkers, some of which may be related to disease. The biomarkers to be used in our somatic mutability and molecular oncology studies are listed in Table 1, together with possible use of stored biological materials. Our somatic mutability study is positioned to look at the systemic effects of radiation. In fact, the observed relationship between GPA Mf, radiation dose, and solid cancers suggests that atomic radiation might exert long-lasting and systemic effects on the mutability of tissue cells of the body and not exclusively of hematopoietic cells, since increased GPA Mf at high doses was closely associated with cancers of various organs and not just the hematopoietic system. We anticipate that the results of the γH2AX and reticulocyte micronucleus assays will deepen our mechanistic understanding of radiation-induced somatic mutability.

Nevertheless, we still are looking at only the “shadow” of radiation effects. This is due, in part, to the fact that our survey has been and will continue to be performed well over 40 years after the bombings, and that the direct targets of radiation are stem/progenitor cells, the nature of which generally differs from that of differentiated cells. In the future, somatic mutability studies need to combine with stem cell biology studies, so that radiation effects on stem cells and early progenitors can be investigated in terms of genetic and epigenetic alterations, stem cell senescence, and genome maintenance.

Carcinogenic pathways appear to differ significantly between organ systems and specific types of pathologies, as evidenced by the markedly different cancer risks in various organs of the A-bomb survivors. Only a few molecular oncology studies on cancers among A-bomb survivors have been reported in the past. This is due in part to the difficulty in collecting cancer tissue samples as well as the difficulty in analyzing those long-term preserved formalin-fixed samples at the molecular level. As a result of our molecular oncology study, sophisticated methods have been developed for the analyses of archived, often decades old, tissue samples [41], and have examined the molecular events in the carcinogenic pathways of particular cancers, as influenced by radiation exposure. Results of our adult-onset papillary thyroid and colorectal cancer studies have suggested that radiation did not alter basic pathways, but preferentially induced specific events: e.g., preferential occurrence of chromosome aberrations in the early stage of papillary thyroid carcinogenesis, and MSI and its related molecular events as consequences, not causes, of colorectal carcinogenesis. Although sufficiently intense ionizing irradiation may be associated with genome-wide hypomethylation, comparable irradiation might be related to hypermethylation of specific genes. This may be the case in MLH1 hypermethylation in colorectal cancer.

Our findings on thyroid and colorectal cancers remain preliminary. Given the potential implications of our preliminary findings, further work is warranted to assess more thoroughly the mechanisms of radiation-associated cancer. To pursue these molecular oncology studies, cancer tissue samples from

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<th>Biomarkers</th>
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<td>Paraformaldehyde- or DMS-fixed blood cells</td>
<td>Somatic mutability</td>
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<td>γH2AX foci</td>
<td>Cultured T-lymphocytes (expanded from cryopreserved uncultured ones)</td>
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<td>RET rearrangements and BRAF mutation</td>
<td>Long-term preserved paraffin blocks</td>
<td>Chromosome aberration vs. point mutation, relative to radiation effects</td>
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<td>MSI-related events (colorectal cancer)</td>
<td>Long-term preserved paraffin blocks</td>
<td>Microsatellite unstable carcinogenesis</td>
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<td>MLH1 gene methylation</td>
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<td>Alterations in Ras signaling</td>
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A-bomb survivors, which are precious but presently spread over many hospitals, need to be collected in greater number and with greater efficiency.

We anticipate that our study will be linked to “molecular event-based risk estimation” of radiation-associated cancers, where the dose-risk relationships will be evaluated not only for a particular cancer-site but also for the fraction of this cancer harboring a specific molecular alteration, for example, radiation-induced risk of papillary thyroid cancer with RET/PTC rearrangements and MSI-positive colorectal cancer. These data will contribute not only to the mechanistic understanding of radiation-associated cancers but also to the future prevention of these cancers associated with natural, medical, occupational, and accidental exposure to radiation.

Acknowledgements

The Radiation Effects Research Foundation is a private non-profit foundation funded equally by the Japanese Ministry of Health, Labour and Welfare and the U.S. Department of Energy, the latter through the National Academy of Sciences. This study is in part supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan. This publication was based on RERF Research Protocols RP3-87, RP1-93, RP5-02, B38-04 and B44-06.

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