# Individual Variation of Somatic Gene Mutability in Relation to Cancer Susceptibility: Prospective Study on Erythrocyte Glycophorin A Gene Mutations of Atomic Bomb Survivors

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## Abstract

**It has previously been reported that hemizygous mutant fraction (Mf) at the glycophorin A (*GPA*) locus in erythrocytes increased with radiation dose in heterozygotes among Hiroshima and Nagasaki atomic bomb survivors. In the present study, we analyzed the relationship between GPA Mf and cancer risk using newly developed cancers among previously cancer-free subjects whose GPA Mf had been measured between 1988 and 1996. Among 1,723 survivors (1,117 in Hiroshima and 606 in Nagasaki), we identified 186 subjects who developed a first cancer by the end of 2000. We compared the radiation dose responses of GPA Mf between cancer and cancer-free groups using a linear-quadratic model fit by multiple regression analysis in combination with age, sex, and city. The slope of the GPA Mf dose-response curve was significantly higher in the cancer group than in the cancer-free group among Hiroshima subjects. Moreover, no significant difference of GPA Mf between cancer and cancer-free groups was found in unexposed controls in the two cities. The same conclusions were obtained using a linear dose-response model and by further analysis using Cox regression of cancer incidence. These findings suggest that there might be interindividual variation in mutability of somatic genes and that Hiroshima survivors who have higher mutability in response to radiation exposure would be expected to have a higher probability of suffering radiation-related cancer.** (Cancer Res 2005; 65(12): 5462-9)

## Introduction

Interindividual variability in human responses to mutagen exposures, including ionizing radiation, is believed to be a critical element in determining individual risk of cancer as well as the incidence of cancer in a population. At least a part of such interindividual variability of cancer susceptibility may be attributed to capacity of responses to oxidative DNA damage generated by mutagens (1). Multistaged defense mechanisms may exist in the responses to oxidative DNA damage, involving the initial defense against reactive oxygen species by superoxide dismutase and catalase, inhibition of incorporating oxidized bases into DNA by hydrolase, and repair of DNA damage (i.e., base excision repair, transcription-coupled repair, global genome repair, mismatch repair, translesion synthesis, homologous recombination, and nonhomologous end joining). Among them, much attention has been paid to several DNA repair genes. There is increasing evidence that mild reductions in DNA repair capacity, assumed to be the consequence of common genetic variation, affect cancer predisposition (2, 3). Currently, molecular epidemiologic studies are being conducted in many laboratories to define the roles that polymorphisms in DNA repair genes play in individual cancer susceptibility (3–5). In contrast to such genetic markers, phenotypic markers of DNA repair capacity and cancer susceptibility comprise both genetically and environmentally determined fractions and express integrated effects of complicated processes where a number of gene products are involved. Thus, phenotypic markers have often played vital roles in cancer research, specifically in prospective cohort studies, assessing the exposure levels (biodosimetry) as well as cancer risk (2, 6).

Many phenotyping assays have been developed using blood cells and skin fibroblasts for quantifying *in vivo* somatic mutations and *in vitro* DNA repair capacity (6). The erythrocyte glycophorin A (GPA) mutation assay, which can enumerate hemizygous mutants at the *GPA* locus in long-lived hematopoietic stem cells of heterozygous donors, provides one useful phenotypical end point for the assessment of cancer risk. This was supported by the findings that highly elevated GPA mutant fractions (Mf) were detected in patients with cancer-prone diseases, such as ataxia telangiectasia (7), Bloom's syndrome (8, 9), Fanconi's anemia (8, 10), and Werner syndrome (11, 12). These patients have defects in genes that are involved in several pathways of DNA repair mechanisms. It was also reported that GPA Mfs can be used as an assessment marker for the development of secondary induced leukemia in patients treated for childhood acute lymphoblastic leukemia (13). These findings suggest that the GPA Mf may, in some way, reflect individual repair capacity and cancer risk.

To clarify the association between radiation-induced mutation and cancer risk, prospective studies are critical to exclude the role of cancer itself in the association, such as through chemotherapy and radiation therapy. Because the atomic bomb (A-bomb) survivor population is an epidemiologically well-controlled cohort in terms of dose estimation (14) and cancer follow-up (15), such an analysis is feasible in this population. We previously measured hemizygous GPA Mf in ~1,200 heterozygous A-bomb survivors in Hiroshima and Nagasaki between 1988 and 1993 and analyzed the dose response of GPA Mf and the relationship between GPA Mf and cancer risk (16). It was found that the doubling dose of GPA Mf was similar to that of solid-cancer incidence in A-bomb survivors. Furthermore, the dose response was significantly higher in persons who had been diagnosed with cancer than in cancer-free

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individuals among Hiroshima survivors. This suggests an earlier onset of cancer due to enhanced mutagenesis or a higher radiation sensitivity in the cancer group. However, although we attempted to exclude all survivors who had undergone chemotherapy and radiotherapy, we may have missed some of them due to incompleteness of the medical records. Thus, because we could not completely exclude the possible effect of the therapies on GPA Mf, a prospective study was desired.

We have extended the GPA Mf measurements to ~1,900 survivors in total as of 1996 and followed them until 2000 to identify newly developed cancers among the previously cancer-free subjects. Based on these prospective data, we reanalyzed the relationship between GPA Mf and cancer development. In the present report, we show the reproducibility of the previous findings and discuss interindividual variation of susceptibility to radiation-induced mutagenesis, which may be associated with subsequent cancer risk.

## Materials and Methods

**Study subjects.** Blood samples were obtained randomly from 1,902 survivors whose MN blood types were heterozygous by the hemagglutination test, who were participating in the Radiation Effects Research Foundation (RERF) Adult Health Study from June 1988 to August 1996. We excluded 179 survivors who were diagnosed with cancer before the GPA measurements and observed subsequent cancer development. Subject ages ranged from 43 to 100; mean ages were 63 for males and 67 for females.

Survivors in Hiroshima and Nagasaki, who have been diagnosed with malignant tumors ($n$ = 186; 118 in Hiroshima; 68 in Nagasaki) through December 2000, were identified from the RERF tumor registry (17). Diagnoses and medical treatment histories for these survivors were also confirmed from the Adult Health Study medical charts. Identified malignant tumors included stomach ($n$ = 32), colon ($n$ = 31), lung ($n$ = 19), liver ($n$ = 18), breast ($n$ = 10), rectum ($n$ = 10), pancreas ($n$ = 8), prostate ($n$ = 7), gall bladder ($n$ = 6), esophagus ($n$ = 5), thyroid ($n$ = 5), and other ($n$ = 35) cancers.

The distribution of subjects by DS86 bone marrow doses (14), sex, and city are shown in Table 1. This distribution is similar to that of the total Adult Health Study population. The estimated dose includes both neutron and γ-ray components. The analyses described in the present report were based on weighted bone marrow doses computed as the γ dose plus 10 times the neutron dose and adjusted for the effect of imprecision on regression analyses (18). The weighting factor will be called the relative biological effectiveness of neutrons, and weighted doses are expressed in sieverts (Sv). This study population consists of Hiroshima and Nagasaki survivors who were exposed to significant radiation doses of ≥0.004 Sv because of their location within 2 km of the hypocenter plus a second group whose exposures were at distances in excess of 3 km from the hypocenter and as a result led to them receiving radiation doses of <0.004 Sv (i.e., doses that are indistinguishable from background). The latter group of distally exposed survivors includes the most appropriate controls for all of our studies of the effects of A-bomb radiation exposures, including the present one.

**Measurement of glycophorin A mutation frequency.** Using the GPA mutation assay, four types of mutant cells, Mϕ, Nϕ, MM, and NN cells, can be detected among the erythrocyte populations of MN heterozygous donors. Hemizygous Mϕ and Nϕ cells are caused by deactivation of $N$ or $M$ alleles of the *GPA* gene, respectively. Homozygous MM and NN cells may be induced by somatic recombination between the two chromosomes on which the $M$ and $N$ alleles reside. Among these four types of mutants, the reproducibility of NN cells was low, probably due to carbohydrate modification of the GPA molecules (19, 20). Also, MM mutant frequency is significantly affected by overlapping of Mϕ mutants in the MM mutant window on the flow cytogram, particularly for the high-dose exposed who have high Mϕ Mfs. Thus, in this report, statistical analysis was undertaken for the mean of Mϕ and Nϕ hemizygous Mf (GPA Mf).

The detailed method for the flow cytometric measurement of mutant erythrocytes has been described previously (20). Briefly, using a single-beam cell sorter, FACStar (Becton Dickinson Immunocytometry System, San Jose, CA), four types of variants lacking the expression of one *GPA* allele were distinguished from normal MN heterozygous cells. By two-color staining with the GPA (M + N)–specific monoclonal antibody (mAb) 10F7 and the GPA (M)–specific mAb 6A7, two mutant cell types, hemizygous Nϕ and homozygous NN cells, from MN heterozygous donors can be detected simultaneously. By combining the GPA (M)–specific mAb 9A3 and the GPA (N)–specific mAb NN3, hemizygous Mϕ and homozygous MM cells can be measured simultaneously. 10F7 and 9A3 mAbs were directly labeled with fluorescein and mAbs 6A7 and NN3 were conjugated with biotin followed by labeling with streptavidin-conjugated phycoerythrin (Biomeda, Foster City, CA). Mutant cells displaying a hemizygous or homozygous phenotype were sorted onto a glass slide. Cells showing typical erythrocyte morphology with fluorescein fluorescence matched for the mutant phenotype were counted under a fluorescence microscope. Typically, ~$10^6$ total erythrocytes were assayed per sample.

**Table 1.** Distribution of subjects excluding persons who had malignant cancer before GPA Mf measurement

| City | Sex | No. subjects | Survivor bone marrow dose (Sv, neutron RBE = 10) | | | | |
|---|---|---|---|---|---|---|---|
| | | | <0.004* | 0.004-0.499 | 0.500-0.999 | 1.000-1.499 | 1.500+ |
| Hiroshima | Male | 360 (54)† | 129 (22) | 132 (13) | 47 (12) | 32 (3) | 20 (4) |
| | Female | 757 (64) | 311 (19) | 292 (25) | 93 (9) | 29 (7) | 32 (4) |
| | Total | 1,117 (118) | 440 (41) | 424 (38) | 140 (21) | 61 (10) | 52 (8) |
| Nagasaki | Male | 221 (36) | 84 (14) | 53 (7) | 45 (7) | 25 (5) | 14 (3) |
| | Female | 385 (32) | 168 (8) | 88 (10) | 77 (8) | 38 (2) | 14 (4) |
| | Total | 606 (68) | 252 (22) | 141 (17) | 122 (15) | 63 (7) | 28 (7) |
| Total | Male | 581 (90) | 213 (36) | 185 (20) | 92 (19) | 57 (8) | 34 (7) |
| | Female | 1,142 (96) | 479 (27) | 380 (35) | 170 (17) | 67 (9) | 46 (8) |
| | Total | 1,723 (186) | 692 (63) | 565 (55) | 262 (36) | 124 (17) | 80 (15) |

Abbreviation: RBE, relative biological effectiveness.
*According to the DS86 dosimetry system, survivors whose dose estimation would result in a free-in-air kerma <5 mGy were automatically assigned doses of zero. However, most of the persons in this category were too far from the hypocenter to have received significant radiation exposure.
†Numbers of persons with cancer diagnosed subsequent to GPA Mf measurement are in parentheses.

**Statistical analysis.** Dose responses for GPA Mf and incidence proportion of cancer were fit using ordinary (least squares) regression. Weighted, adjusted bone marrow dose was used as described above. Age at examination was centered at its mean (65 years). City, sex, and cancer status were treated as indicator variables. Application of least-squares regression to the GPA Mf and radiation dose-response data for purposes of statistical testing would necessitate logarithmic transformation of both the GPA Mf and radiation dose variables (16) to achieve approximate normality and constant variance of the response variable (log GPA Mf) and approximately uniform distribution of the predictor variable (log radiation dose), but we desired to mimic standard radiobiological practice and fit linear or linear-quadratic dose responses. Thus, we did not transform the variables for the purpose of estimating the radiation dose response and instead verified the fit of the least-squares regression using nonparametric curve-fitting methods. As a further check on adequacy of the dose-response fit, individual observations with large influence on the regression analysis were identified through single-deletion regression diagnostics and regression models were refit after excluding such points. As a result, the small number of subjects with GPA Mf values >400 was not used in estimating the GPA Mf dose response. For fitting the cancer incidence proportion to radiation dose, least squares regression was applied to the binary indicator of cancer status. Approximately homogeneous variance and fit to the data were confirmed by comparing the fitted regression to a plot of binomial proportions grouped on radiation dose with approximately equal numbers of subjects (Fig. 1).

Follow-up for incident cancer subsequent to GPA measurement was analyzed using Cox regression with age as the time scale and adjustment for year of birth and age at examination. The effect of GPA Mf on cancer incidence was assessed using either the logarithm of continuous GPA Mf to reduce the influence of the small number of points with large values of GPA Mf, or the untransformed GPA Mf excluding the subjects with values larger than 400. For graphic presentation of the results of the Cox regression, summary plots of cumulative incidence (proportion of subjects who were free of cancer) were produced by dividing subjects into two strata—low and high values of GPA Mf—based on the median GPA Mf among cancer cases.

All analyses were conducted using S-plus version 2000 (Mathsoft, Inc., Seattle, WA).

## Results

**Cancer prevalence in the study cohort.** One hundred eighty-six subjects developed a first cancer between the GPA Mf measurement and the end of 2000. There was a statistically significant increase in cancer incidence proportion with dose after adjustment for city, sex, and age (Fig. 1). This suggests that the subjects of the present study developed cancers in a dose-dependent manner even >40 years after exposure.

**Effects of cancer status and city difference on dose responses of glycophorin A mutant fraction.** Figure 2 shows nonparametric curves for the GPA Mf (the mean of hemizygous Mϕ and Nϕ Mf) values according to bone marrow dose among all study subjects ($n$ = 1,902), including cancer cases diagnosed before GPA Mf measurements. The plots suggest that the GPA radiation dose response is steeper among cancer patients in Hiroshima, particularly among those whose cancer was diagnosed subsequent to GPA measurement (Fig. 2, *left*). Persons who had cancer diagnosed before the time of GPA measurement may not be representative, because some individuals with cancer might have been censored—debilitated or deceased—and unable to attend the Adult Health Study examination. In contrast, no apparent differences were observed between cancer and noncancer groups among Nagasaki survivors (Fig. 2, *right*).

To evaluate further the possible difference in cancer-related GPA dose response between the two cities, we did standard regression on GPA Mf using bone marrow dose, cancer status, sex, and age at examination as covariates, excluding subjects with values of GPA Mf >400 and those who had cancer diagnoses before GPA measurement (Table 2). Background GPA Mf (estimate for 0 Gy exposure) was lower in Nagasaki than in Hiroshima and lower in females than in males. An increase in GPA Mf with age of the subject at examination was only marginally significant, probably because age-dependent increase in GPA Mf reaches a plateau after about 50 years of age (21). There was an initial increase in the GPA response with bone marrow dose, followed by an attenuation in the slope (negative quadratic term). Although the quadratic term was statistically significant ($P$ = 0.033), there was little quantitative difference in the results with or without the quadratic term except for a slightly lower dose-response slope without the quadratic term. There was no significant difference in background GPA Mf between male subjects who subsequently developed cancer and those who did not, but among females the background GPA Mf was lower among those with cancer.

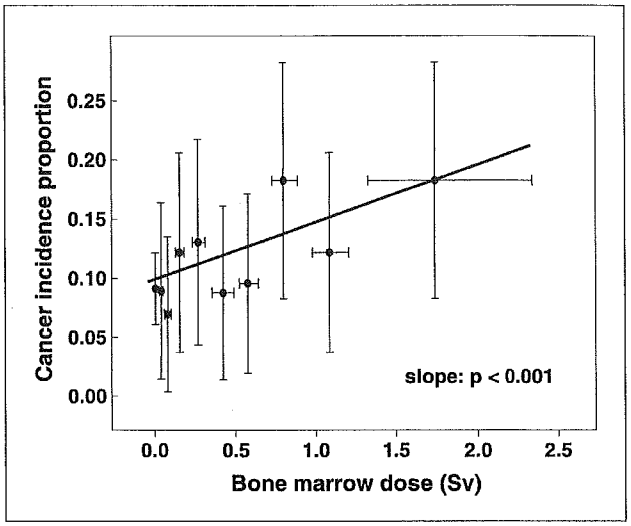


**Figure 1.** Dose response of cancer incidence proportion subsequent to GPA Mf measurement adjusted for city, sex, and age at examination. The total number of subjects is 1,723. *Points*, crude proportions in 10 dose groups with approximately equal numbers of subjects ($n$ = 115) except for unexposed controls ($n$ = 691); *bars*, SE of the estimated proportions and the quartiles of dose.

The fitted linear-quadratic regression models for each of the two cities, adjusted for sex and age, are shown in Fig. 3. The dose response of GPA Mf was significantly higher in Hiroshima subjects who subsequently developed cancer than in those who did not. Whether there was a difference in dose response by cancer status depended significantly on city (a three-way interaction between city, dose, and cancer status; $P$ = .0081), with no difference in Nagasaki. Seven points were identified that had a high influence on the value of the city × cancer × dose three-way interaction term. Upon deleting these points, the value of the interaction term decreased somewhat but remained statistically significant. Among Nagasaki subjects, the initial slope of the GPA dose response in the linear-quadratic model adjusted for the average values of the other factors was 22.6 in cancer-free subjects and 22.3 in subjects with subsequent cancer ($P$ = 0.29). In Hiroshima, the similarly adjusted initial slopes were 35.8 in cancer-free subjects and 51.3 in subjects with subsequent cancer ($P$ = 0.0039). There was no evidence that the quadratic term of the dose response differed according to cancer status.

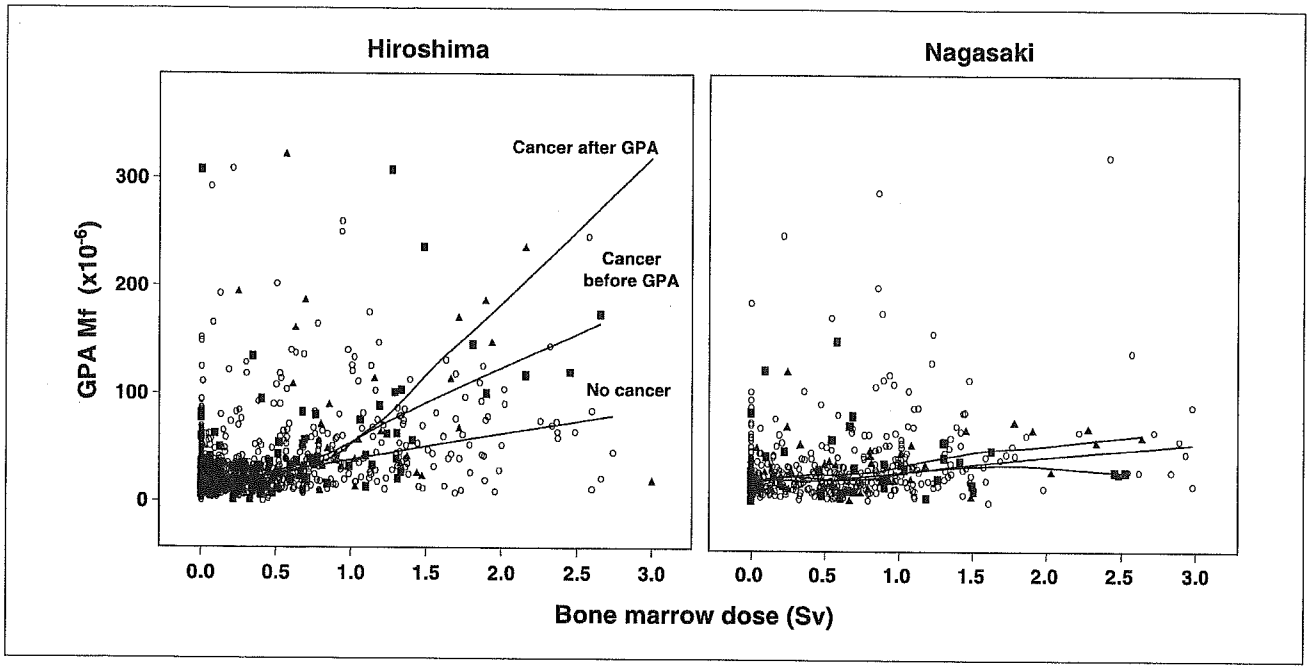


**Figure 2.** Dose response of GPA Mf based on nonparametric curve fitting for cancer (either before or after GPA Mf measurement) and noncancer groups in Hiroshima and Nagasaki. Symbols indicate subjects who were diagnosed with cancer before GPA Mf measurement (■), those who were diagnosed with cancer after GPA Mf measurement (▲), and those who were not diagnosed with cancer (○). Lines denote fitted dose-response curves for cancer and noncancer groups.

**Difference of glycophorin A mutant fraction between cancer and noncancer groups among high dose–exposed subjects.** Because the GPA Mf values are highly skewed, even when restricted to $\leq 400 \times 10^{-6}$, the results of statistical tests of parameters from the regression analysis may not be reliable.

**Table 2.** Regression analysis of GPA Mf using linear and linear-quadratic dose-response models

| Term | Estimated value ± SE [$\times 10^{-6}$] ($P$ value) | |
|---|---|---|
| | Linear model | Linear-quadratic model |
| City (Nagasaki) | −7.1 ± 3.0 (0.018) | −7.5 ± 3.0 (0.013) |
| Sex (female) | −6.5 ± 2.1 (0.0016) | −6.4 ± 2.1 (0.0020) |
| Age at exam (per year) | 0.13 ± 0.076 (0.078) | 0.13 ± 0.076 (0.099) |
| Dose (initial slope, per SV) | 26 ± 2.0 (<0.0001) | 32 ± 3.5 (<0.0001) |
| Dose (quadratic term) | — | −3.6 ± 1.7 (0.033) |
| Cancer (present) | 5.7 ± 4.6 (0.21) | 5.5 ± 4.6 (0.23) |
| Sex-cancer interaction | −12 ± 4.8 (0.013) | −12 ± 4.8 (0.012) |
| City-dose interaction | −5.5 ± 3.1 (0.071) | −5.0 ± 3.1 (0.10) |
| Cancer-dose interaction | 15 ± 5.3 (0.0049) | 15 ± 5.3 (0.0038) |
| City-dose-cancer interaction | −21 ± 7.9 (0.0068) | −21 ± 7.9 (0.0081) |

NOTE: Persons with GPA Mf >400 × $10^{-6}$ were excluded.

Therefore, we compared log-transformed GPA Mf values between high dose and unexposed persons, thereby avoiding assuming any particular dose-response model, using either a $t$ test (Fig. 4) or regression analysis with adjustment for sex and age (Table 3). Among unexposed subjects, there was no difference in log GPA Mf between persons with and without cancer in either city. The log GPA Mf was significantly higher in subjects with subsequent cancer than without cancer among the heavily exposed (>1.5 Sv) subjects in Hiroshima ($t$ test $P = 0.012$, regression $P = 0.0057$) but was not significantly different between subjects with and without cancer in Nagasaki ($t$ test $P = 0.52$, regression $P = 0.21$).

The analysis of cancer onset rates during the follow-up period by Cox regression confirmed the finding that cancer risk was related to GPA Mf level among high-dose–exposed subjects in Hiroshima but not in Nagasaki (Fig. 5). Whereas there was no association between log GPA Mf level and cancer onset rate among unexposed persons or Nagasaki high-dose–exposed persons, there was significantly higher risk of cancer with higher GPA Mf value in Hiroshima high-dose–exposed persons ($P = 0.043$). The estimated relative risk of cancer for a 10-unit difference in GPA Mf (i.e., a 50% increase over the median) was 1.13 (95% confidence interval, 1.00-1.27; Table 4). Relative risks of cancer for GPA for all individual dose groups by city are shown in Table 4. There was no change in the significance of the results when log GPA was used.

The $t$ tests and Cox regression analyses were repeated after excluding persons whose follow-up was <1 year. There was no change in the pattern of results. The association among high dose and cancer on log GPA Mf was significant in Hiroshima ($P = 0.024$) but not in Nagasaki ($P = 0.41$). The effect of GPA Mf on cancer incidence was significant in high-dose persons in Hiroshima ($P = 0.041$) but not in Nagasaki ($P = 0.66$).

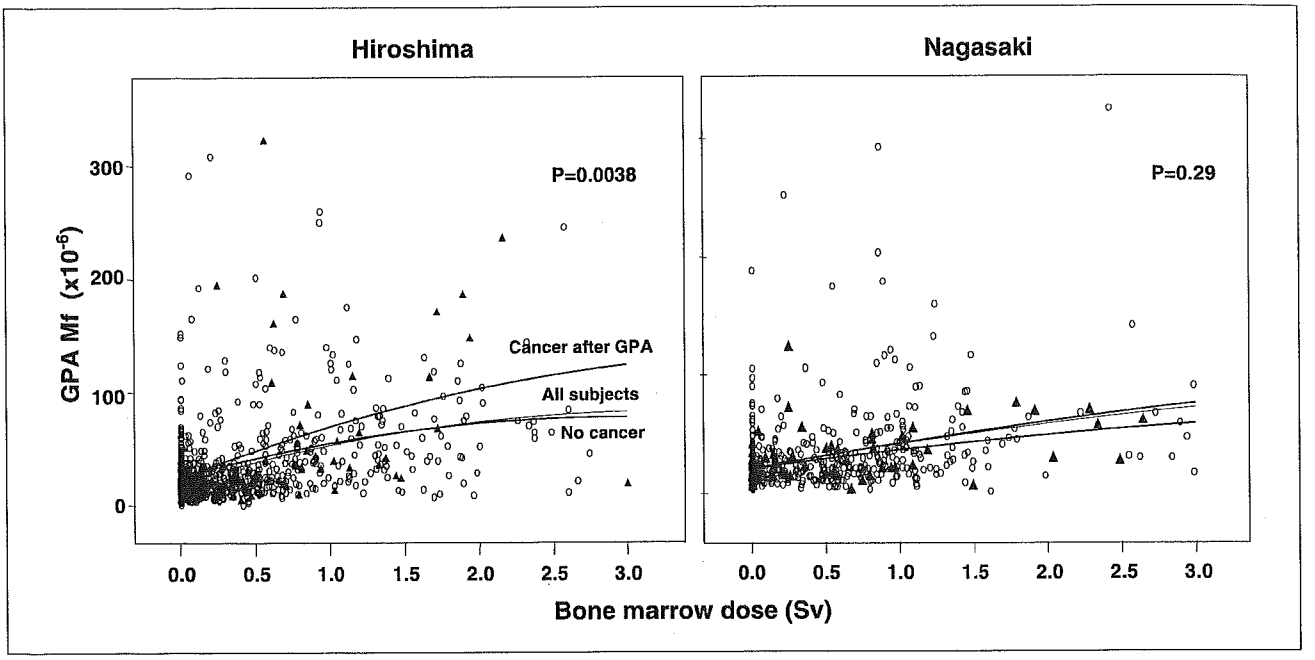


**Figure 3.** Dose response of GPA Mf among cancer (post-GPA Mf measurement) and noncancer groups based on the linear-quadratic regression model in Hiroshima and Nagasaki. The number of subjects in each group is listed in Table 1. Symbols indicate subjects who were diagnosed with cancer after GPA Mf measurement (▲) and subjects without cancer (○). Lines denote fitted dose-response curves for cancer and noncancer groups. *P* values represent the statistical significance of difference of dose response (quadratic term) between cancer post-GPA Mf measurement and noncancer groups.

## Discussion

It is increasingly accepted that the accumulation of multiple abnormalities in cancer-associated genes of a target cell is required for the development of cancer (22–24). Although the mechanism of radiation carcinogenesis is still unknown, some evidence has been presented that A-bomb radiation seems to reduce the number of gene changes needed for cancer induction, thereby inducing earlier onset of cancer in the exposed, compared with the unexposed (25). If GPA Mf reflects nonspecific mutability of all somatic cells in an individual, it can be presumed that GPA Mf may also reflect the prevalence of mutations at cancer-associated genes. Among survivors exposed to the same dose, those who have a higher GPA Mf would be expected to have a higher probability of suffering cancer at any given point in time. In fact, as shown in the present study, the dose response of GPA Mf in the cancer group among Hiroshima survivors was found to be significantly higher than that in the noncancer group. The dose response among persons with cancer diagnosis before GPA measurement was intermediate to that of the groups with no cancer or cancer after GPA measurement, which is further consistent with a higher cancer risk for high GPA Mf radiation–exposed subjects because persons at high risk of cancer would more likely be censored (unobserved due to death or debility) before GPA measurement. The individual difference of GPA Mf in the same dose group might be explained by individual variation in the capacity to repair radiation-induced DNA damage. We discuss below the validity and feasibility of these hypotheses regarding individual differences in DNA repair capacity among A-bomb survivors.

A potentially important source of interindividual variability in relation to cancer risk is DNA repair capacity, including the DNA repair–defective cancer-prone diseases, such as ataxia telangiectasia, Bloom's syndrome, Fanconi's anemia, and Werner syndrome. Apart from these rare and extreme familial cases, there is increasing evidence that a moderate reduction in DNA repair capacity contributes to the sporadic incidence of cancer in the general population (2, 3). Conventional phenotype assays have detected considerable interindividual variation in DNA repair capacity (2, 26, 27). These reduced repair capacity phenotypes have been associated with an increased risk of cancer (2, 28, 29). Evidence of the importance of moderate reduction in DNA repair capacity is also accumulating from mouse models, which have provided results regarding cancer risk increased by heterozygous knock-out in DNA repair genes (30, 31) and those regarding strain differences in cancer susceptibility (32, 33). Furthermore, a number of molecular epidemiologic studies have been initiated using the data from systematic screening of populations for common variants in DNA repair genes (3, 34). Associations of common variants in several repair genes with increased cancer risk have been reported in case-control studies (5). In general human populations, it has been suggested that individual differences in peripheral blood T cell chromosome aberration frequencies may be associated with individual differences in cancer susceptibility (35, 36). These accumulating data are consistent with the hypothesis that interindividual variation in DNA repair capacity has an impact on cancer risk.

Statistical analysis in this study showed that there was a city difference (i.e., significant interaction among radiation, cancer, and city in their association with GPA MF; Table 2). This does not necessarily imply that there is a significant correlation between GPA Mf dose response and cancer in Hiroshima subjects but not in Nagasaki subjects. Small numbers of cases in Nagasaki make it difficult to clearly state the apparent negative finding. However, the observed city difference may be, at least in part, due to possible differences in ethnic background between Hiroshima and Nagasaki, which were suggested by the previous biochemical genetic study of

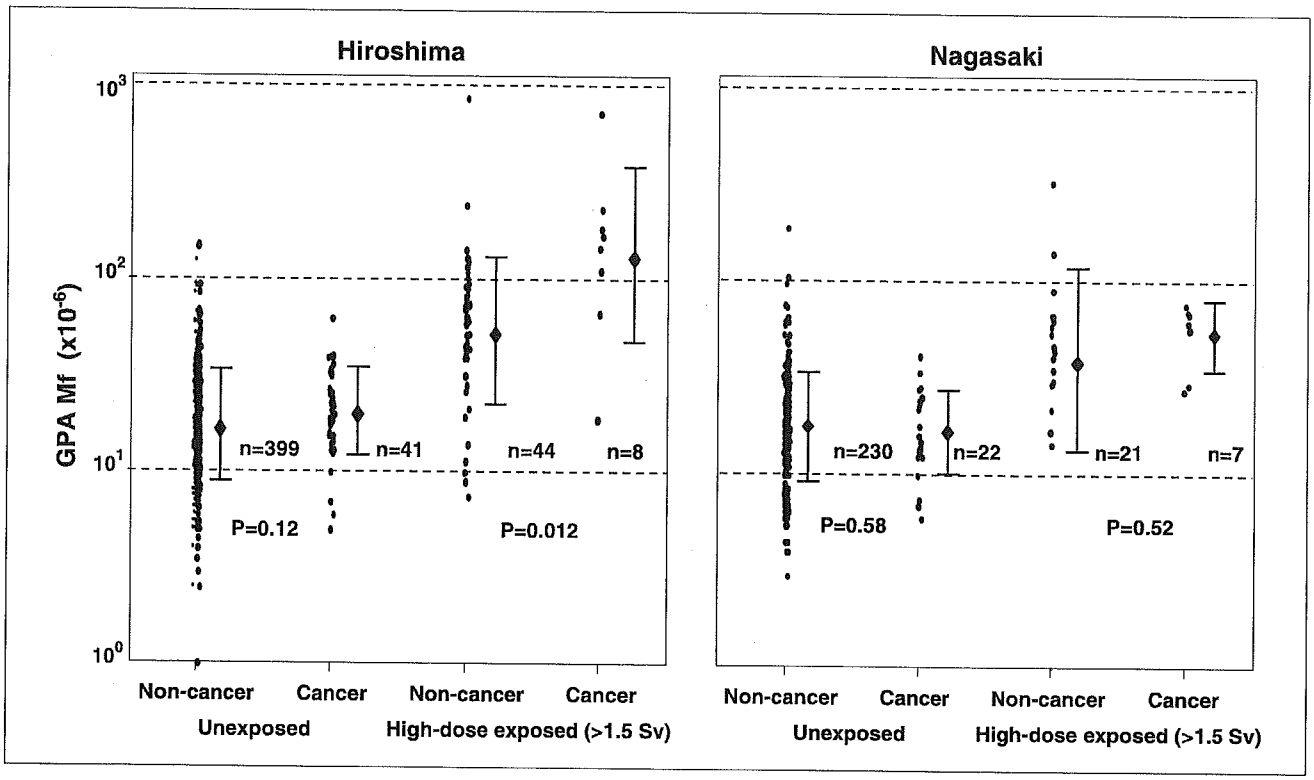


**Figure 4.** Comparison of GPA Mf between cancer and noncancer groups within unexposed and high dose exposed (>1.5 Sv), Hiroshima and Nagasaki. ◆, mean of GPA Mf; *bars*, SE. *P* values represent the statistical significance of difference of GPA Mfs between cancer and noncancer groups within each dose group.

A-bomb survivors and their children (37). We might presume that the proportion of individuals who have higher mutability of somatic genes is somewhat larger in Hiroshima than in Nagasaki. Interestingly, the background GPA Mf of Hiroshima is significantly higher than that of Nagasaki, as shown in the present study. Such a city difference was also reported for the background solid-tumor mortality (38) and chronic myelogenous leukemia (CML) of survivors (39). City differences of dose response (lower in Nagasaki) were also suggested for the solid tumor mortality (15), the chromosome aberration frequencies in lymphocytes (40), and the incidence of CML (39). These city differences have been attributed to dose estimation errors and/or to a qualitative difference in the radiation produced by the bombs (i.e., the difference in the amount of neutron and γ-ray components), assuming that there is no city difference in radiation sensitivity of A-bomb survivors (15, 40). Because recent genomic analyses have shown extensive interindividual—including ethnic—variations in gene polymorphisms, as mentioned above, this assumption should be reassessed.

**Table 3.** Relationship between log GPA Mf and cancer status in high dose–exposed survivors

| City | Term | Estimated difference in log GPA Mf | SE | *P* |
|---|---|---|---|---|
| Hiroshima | Exposed | 0.503 | 0.0477 | <0.0001 |
| | Cancer | 0.0368 | 0.0495 | 0.46 |
| | Exposure-cancer interaction | 0.342 | 0.123 | 0.0057 |
| Nagasaki | Exposed | 0.365 | 0.0670 | <0.0001 |
| | Cancer | −0.0622 | 0.0634 | 0.33 |
| | Exposure-cancer interaction | 0.177 | 0.141 | 0.21 |

NOTE: Results of fitting log GPA Mf, comparing high dose (>1.5 Sv) with nonexposed with adjustment for sex and age at examination.

The following possible caveats of the present study should be kept in mind. We conducted a prospective study, which is critical to exclude possible effects of chemotherapy and/or radiotherapy on *in vivo* somatic mutations. Nevertheless, because the period between GPA Mf measurement and cancer diagnosis is rather short (average: about 3.7 years; range: 22 days-9.4 years; $n$ = 187), it is possible that tumors of preclinical size had already developed before GPA Mf measurement. Tumor burden, even with a very small lesion, might increase somatic mutations through high metabolic rate and excessive endogenously generated oxidative stress. However, this may not be the case because GPA Mf values were nearly constant in the cancer subjects ($n$ = 29) whose Mf were measured more than twice during the 8-year examination period before cancer diagnosis (data not shown). Further, the radiation dose responses of GPA Mf between cancer and cancer-free groups did not change after excluding all persons whose follow-up ended within 1 year of GPA measurement (data not shown). Another factor complicating the interpretation is uncertainty in dose estimation, because persons with radiation-related outcomes, such as cancer, are more likely to have underestimated dose than persons without. However, our comparison between high-dose-exposed

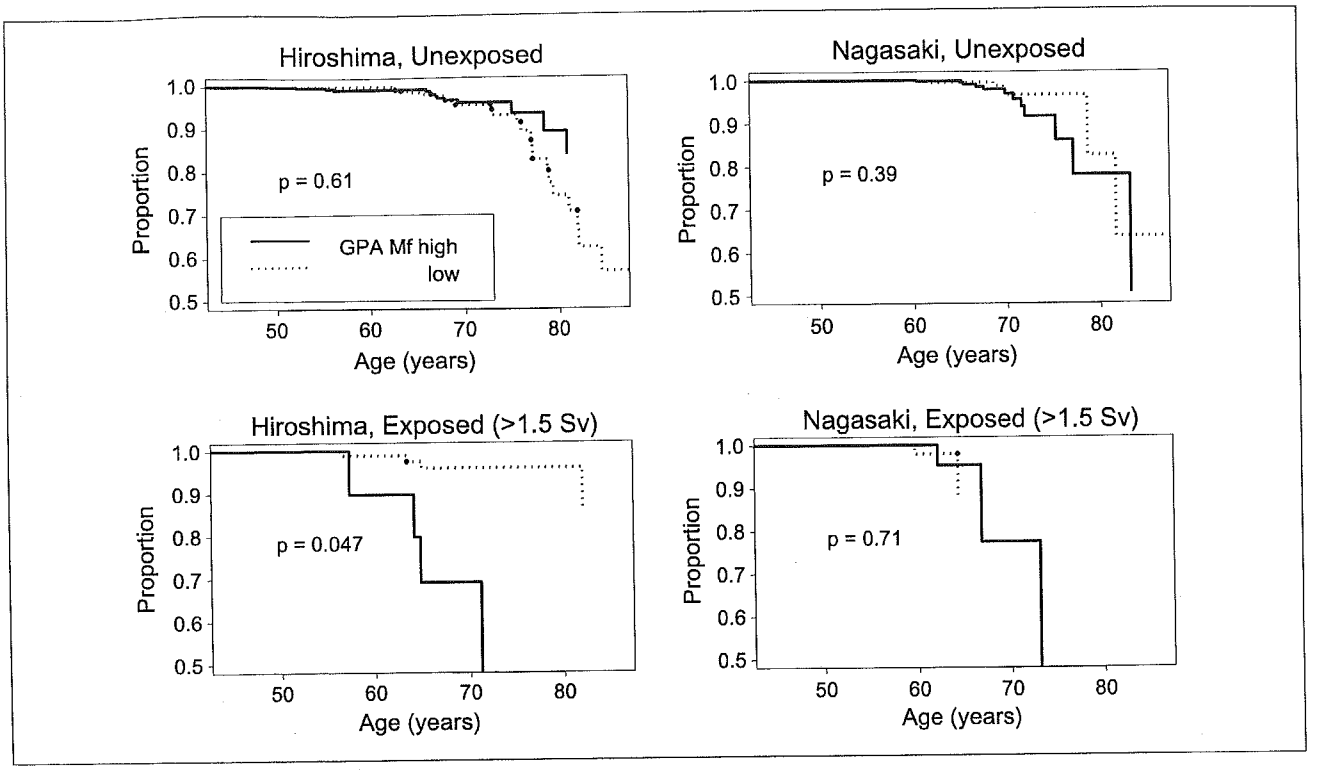


**Figure 5.** Cox regression analyses of cancer incidence following GPA Mf measurement within unexposed and high dose exposed (>1.5 Sv) in Hiroshima and Nagasaki. *Dashed line*, low GPA Mf; *solid line*, high GPA Mf; low and high are defined as below or above the median GPA Mf level among cancer cases. Numbers of cases of cancer are as shown in Fig. 4. *P* values represent the statistical significance of difference of cancer incidence between high and low GPA Mf groups within each dose group.

and nonexposed persons, which is unaffected by dose uncertainties, did not change the conclusions.

Although our hypothesis is valid, it is far from proved based on the present study alone. We believe that the evolving ability to study polymorphisms in DNA repair genes can contribute to understanding about the relationship between DNA repair capacity and cancer risk in A-bomb survivors. Because the difference in GPA Mf between cancer and cancer-free groups is significant only for high dose–exposed survivors, a candidate polymorphic gene affecting interindividual variability could be one involved in repair of DNA double-strand breaks induced by high-dose irradiation. Double-strand breaks are potentially cytotoxic to cells and mutagenic. At least two molecular mechanisms are involved in the pathway of double-strand break repair: homologous recombination repair and nonhomologous end joining (3, 5, 41, 42). Homologous recombination repair occurs predominantly in the S or $G_2$ phase of cell division and exchanges DNA strands between the damaged chromatid and the intact sister chromatid. Nonhomologous end joining repair involves

**Table 4.** Relative risk of cancer for GPA (relative risk for 10-unit difference in GPA Mf)

| Exposure category | Hiroshima | Nagasaki | Both cities |
|---|---|---|---|
| Zero dose (<0.004 Sv) | 0.96 (0.80, 1.16)<br>*P* = 0.69 | 0.82 (0.57, 1.18)<br>*P* = 0.28 | 0.91 (0.77, 1.07)<br>*P* = 0.26 |
| All exposed (≥0.004 Sv; no dose adjustment) | 1.03 (0.99, 1.08)<br>*P* = 0.15 | 1.00 (0.93, 1.09)<br>*P* = 0.92 | 1.02 (0.99, 1.06)<br>*P* = 0.20 |
| All exposed (≥0.004 Sv; with dose adjustment) | 1.03 (0.98, 1.08)<br>*P* = 0.23 | 0.98 (0.89, 1.08)<br>*P* = 0.68 | 1.02 (0.98, 1.06)<br>*P* = 0.45 |
| Low dose (≤1.5 Sv) | 1.01 (0.95, 1.07)<br>*P* = 0.80 | 0.99 (0.89, 1.10)<br>*P* = 0.81 | 1.00 (0.96, 1.05)<br>*P* = 0.87 |
| High dose (>1.5 Sv) | 1.13 (1.00, 1.27)<br>*P* = 0.043 | 0.98 (0.82, 1.17)<br>*P* = 0.82 | 1.06 (0.98, 1.14)<br>*P* = 0.14 |

NOTE: Relative risk of cancer for GPA is based on Cox regression analysis of cancer risk by age, with adjustment for sex and year of birth, and relative risk for 10-unit difference in GPA Mf is restricted to GPA values of $400 \times 10^{-6}$ and below.

direct ligation of the two double-strand break ends. These two pathways are thought to involve numerous molecules. Although extensive polymorphic variation in double-strand breaks repair genes has been reported, only a few common polymorphisms of these genes have been examined in epidemiologic studies for their association with cancer risk (43–48). We are planning to study the A-bomb survivors further to elucidate the relationship between DNA repair gene polymorphisms and risk of radiation-induced cancer. The phenotypical data, such as those obtained in the present study, will provide valuable information toward drawing conclusions from genotype-cancer association analyses in future studies.

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# Tumor Induction by Azoxymethane (AOM) and 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in F344 Rat Gastric Mucosa Featuring Intestinal Metaplasia Caused by X-irradiation

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Male F344 5-week-old rats were X-irradiated, and 16 weeks after the first dose, azoxymethane (AOM) was injected or 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) was given by intragastric intubation. Tumors in the pylorus of the glandular stomach were observed in 4 out of the 29 animals receiving X-rays + AOM and in 4 out of the 25 animals receiving X-rays + PhIP, 12 months after administration. No such lesions were found in the chemical or X-ray alone groups. Intestinal metaplasia and some induced tumors were positive for CDX2. It was concluded that the presence of intestinal metaplasia may increase sensitivity to the induction of gastric tumors by colon carcinogens.

Key Words: Gastric tumor, Azoxymethane, 2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine, F344 rats, Intestinal metaplasia

Based on investigations in humans, intestinal metaplastic changes in the stomach have been considered precancerous lesions or a predisposing condition for differentiated gastric carcinoma development (1-7). However, we experimentally investigated an inverse relationship between quantity of intestinal metaplasia, with or without Paneth cells, and gastric tumor development, and established that its presence does not exert a positive influence on induction of gastric neoplasia by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or N-methylnitrosoure (MNU) in rats (8,9). The situation is complex, however, because Nakagawa et al. have indicated that colorectal mucosa implanted into the glandular stomach, like the intrinsic large intestine, is sensitive to tumorigenesis caused by the colon carcinogen, 1,2-dimethylhydrazine (DMH), in contrast to normal gastric mucosa (10). Furthermore, we reported that induction of intestinal metaplastic mucosa in the glandular stomach is associated with susceptibility to tumorigenesis due to DMH (11,12).

The present study was designed to further examine whether intestinal metaplasia might be a target for azoxymethane (AOM) or 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)-induction of malignant tumors in the glandular stomach.

## Materials and Methods

*Animals.* Male F344/DuCrj rats, 5 weeks of age at the commencement, were purchased from Charles River and housed five to a polycarbonate cage under constant conditions of temperature (24 ± 2°C) and relative humidity (55 ± 10%), with a 12:12-hour light-dark cycle. The animals were maintained according to the "Guide for the Care and Use of Laboratory Animals" established by Hiroshima University. All rats were provided with a commercial diet (MF; Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*.

The animals were X-irradiated according to the method described previously (8,9,13), with two X-ray doses of 10 Gy each at a three-day interval (total dose, 20 Gy).

Four months after the first irradiation, initiation of azoxymethane (AOM, Sigma Chemical Co., St. Louis, MO) (14,15) or 2-amino-1-methyl-6-pheny-

limidazo [4,5-b]pyridine (PhIP, NARD, Amagasaki, Japan), both of which induce colon cancer (16-19), was commenced. AOM was given in weekly subcutaneous injections of 15 mg/kg body wt for 3 weeks, while PhIP was administered every 2 days, 3 times per week for a total of 10 doses of 75 mg/kg body wt by intragastric intubation.

*Experimental Procedure.* A total of 169 rats was divided into 6 groups. The animals in Groups 1 to 3 were X-ray irradiated. Those in Groups 1 and 4 were given PhIP, while Groups 2 and 5 received AOM. All were fed a normal MF diet throughout the experimental period. The animals were killed and autopsied when they became moribund and all remaining rats were killed by ether anesthesia 12 months after the initial chemical carcinogen treatment. The stomach, and the small and large intestinal tracts were removed, opened and extended on cardboard for inspection. The location of individual tumors was recorded by measuring the distance from the pyloric ring in the small intestine and from the anus in the large intestine. The numbers and sizes of individual tumors were also noted. Whole tissues were fixed in 10% neutral formalin. Alkaline phosphatase (ALP)-positive foci in the gastric mucosa were detected by the naphthol-AS-MX-phosphate-fast blue RR staining method (20) and the numbers of ALP-positive foci in the whole gastric mucosa per rat were counted under a dissection microscope with a double-blind protocol. Sections of paraffin-embedded tissue were routinely stained with hematoxylin and eosin, and for clarification, when necessary, with periodic acid Schiff-Alcian-blue (AB-PAS). Other organs were removed, fixed in 10% neutral formalin and stained with HE.

Intestinal metaplasias were categorized using the following histological criteria (21,22): type A, gastric mucosa with goblet cells which were positive for AB-PAS; type B, intestinal-type crypts without Paneth cells or type C, intestinal metaplasia with Paneth cells (alkaline phosphatase-positive foci). Using these criteria, the numbers of metaplastic crypts were counted separately for 2 sections through the lesser curvature (pylorus) and 4 through the greater curvature (fundus) in a double-blind fashion. Tumors in the stomach, small intestine and large intestine were classified into two types, adenoma, especially stomach, atypical hyperplasia (ATP, shown in Fig.1) and adenocarcinomas invading the musclaris mucosa or further, and also into two histological types, the well-differentiated (Fig.2) and poorly-differentiated types (Fig.3), the latter including both mucinous and signet ring cell forms.

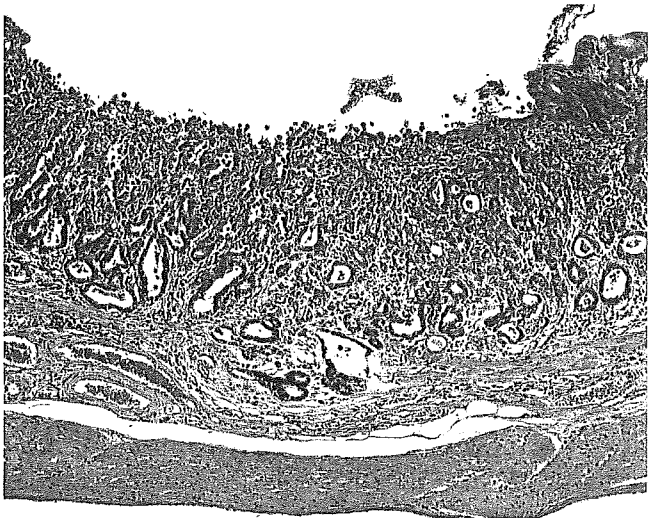

**Fig. 1 -** Atypical hyperplasia shown proliferation of atypical glands in mucosa, x100, HE staining.

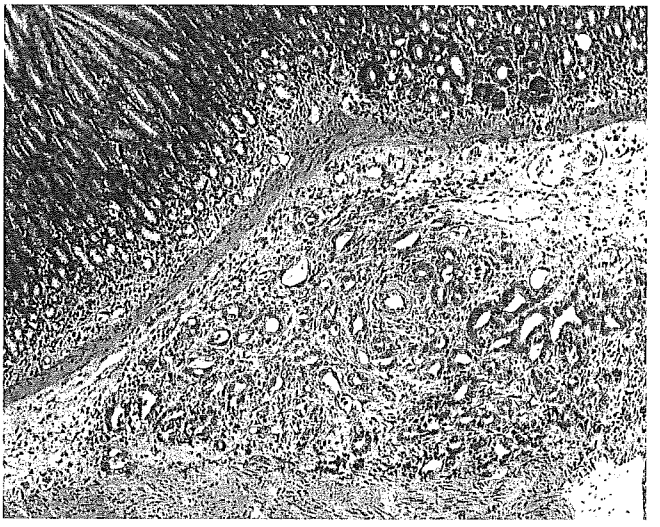

**Fig. 2 -** Well differentiated adenocarcinoma shown atypical glands invaded all the layer of the gastric wall, x100, HE staining.

*Immunohistochemistry.* Paraffin-embedded sections were deparaffinized in xylene, and rehydrated through graded alcohols. A 0.05 M PBS buffer was used to prepare solutions and for washes between the various steps. Incubations were performed in a humidified chamber. Three-μm-thick sections were treated for 30 min at room temperature with 2% BSA and incubated with primary antibodies against CDX2 (diluted 1:50; Biogenex CDX2-88) (23) for 1 hour at

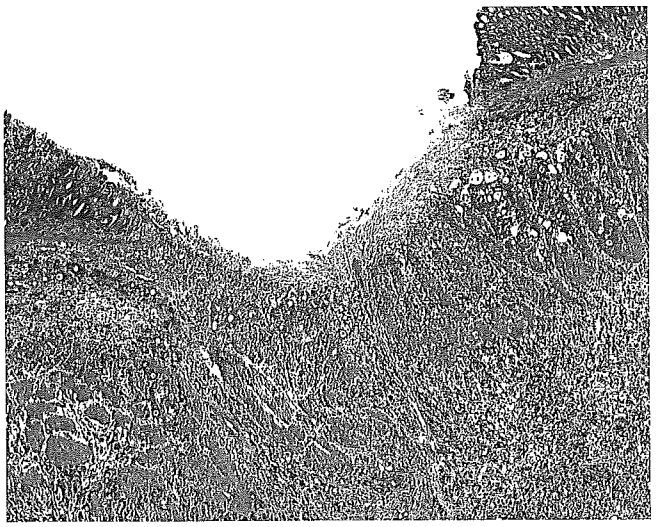

**Fig. 3 -** Signet ring carcinoma, x40, HE staining.

room temperature. For each case, negative controls were performed on serial sections whereby incubation with the primary antibody was omitted. All slides were then exposed to the secondary antibody, biotinylated horse anti-universal-monkey IgG (Vectastain Universal Quick Kit, Vector Laboratories, Ca., Catalog No. PK-8800) and peroxidase-conjugated streptavidin complexes. Peroxidase activity was visualized by treatment with $H_2O_2$ and diaminobenzidine for 5 min. At the last step, the sections were counterstained with hematoxylin for 1 min. CDX2-positive cells were observed.

*Statistical analysis.* The significance of differences in numerical data was evaluated by using the chi-squared test and Student's t test.

## Results

Mean survival did not significantly differ among the groups. Body weights in the chemical carcinogen treatment groups were significantly decreased as compared to those in the control group (Table I). Heart weights were lower in the X-ray + PhIP and X-ray and PhIP groups and liver in the AOM, kidneys in the PhIP and AOM, testes in the AOM and spleens in the X-ray + PhIP and X-ray groups were significantly smaller than those of the controls. On the other hand, spleen weights significantly increased. Relative liver, kidney, and testis weights (relative weight; organ weight/body weight x 1,000) in the X-ray + PhIP group and relative spleen weights in the AOM group were significantly enlarged, while in the X-ray goup they were smaller than those in the controls (Table II).

The incidence and number of areas of intestinal metaplasias in the X-ray irradiated groups were significantly increased as compared to those in the non-irradiated groups (Tables III and IV). Incidences in

**Table I -** Body and organ weights

| Group | Mean survival | Body Weight (g) | Organ weight (g) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Heart | Liver | Kidney | Adrenal | Testis | Spleen |
| X-ray+PhIP | 356±32 | 386±50** | 1.12±0.09** | 11.6±1.5 | 2.47±0.35 | 0.052±0.006 | 3.24±0.18 | 0.60±0.13** |
| X-ray+AOM | 337±50 | 415±37** | 1.19±0.11 | 12.0±3.0 | 2.36±0.21 | 0.084±0.101 | 3.36±0.34 | 0.80±0.42 |
| X-ray | 365±11 | 433±35 | 1.14±0.13** | 11.7±1.1 | 2.37±0.19 | 0.056±0.014 | 3.35±0.27 | 0.63±0.13** |
| PhIP | 364 | 425±26** | 1.14±0.10** | 11.3±1.0 | 2.29±0.23* | 0.063±0.012 | 3.21±0.30 | 0.79±0.10 |
| AOM | 359±10 | 427±23* | 1.21±0.11 | 11.2±1.1* | 2.29±0.14* | 0.055±0.010 | 3.14±0.35** | 1.09±0.19** |
| Control | 364 | 452±33 | 1.26±0.12 | 12.2±1.3 | 2.43±0.20 | 0.065±0.015 | 3.39±0.29 | 0.86±0.10 |

**: Significantly difference from Control value (P<0.05) - **: Significantly difference from Control value (P<0.01)*

**Table II** - Relative weight*

| Group | Heart | Liver | Kidney | Adrenal | Testis | Spleen |
|---|---|---|---|---|---|---|
| X-ray+PhIP | 2.96±0.59 | 30.7±5.9** | 6.56±1.52** | 0.139±0.031 | 8.61±1.92** | 1.59±0.43 |
| X-ray+AOM | 2.88±0.26 | 29.1±8.2 | 5.69±0.38 | 0.199±0.226 | 8.16±1.04 | 1.96±1.14 |
| X-ray | 2.66±0.33 | 27.1±2.4 | 5.49±0.43 | 0.131±0.037 | 7.76±0.61 | 1.47±0.42** |
| PhIP | 2.68±0.18 | 26.7±1.9 | 5.41±0.72 | 0.149±0.031 | 7.58±0.76 | 1.88±0.30 |
| AOM | 2.83±0.23 | 26.1±1.8 | 5.37±0.31 | 0.128±0.021 | 7.35±0.84 | 2.55±0.46** |
| Control | 2.78±0.24 | 26.9±1.7 | 5.39±0.37 | 0.144±0.031 | 7.52±0.68 | 1.91±0.20 |

**:Organ weight/Body weight x 1,000 - **: Significantly difference from Control value (P<0.01).*

**Table III** - Incidence of Intestinal metaplasia (%)

| Group | ALP | Pylorus | | | | Pylorus+Fundus | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | Total | A | B | C | Total |
| X-ray+PhIP | 81 | 32 | 91 | 59 | 95 | 36 | 95 | 50 | 95 |
| X-ray+AOM | 66 | 26 | 78 | 65 | 82 | 26 | 87 | 74 | 87 |
| X-ray | 66 | 0 | 94 | 47 | 94 | 0 | 94 | 50 | 94 |
| PhIP | 0 | 8 | 0 | 0 | 8 | 8 | 8 | 0 | 25 |
| AOM | 0 | 17 | 8 | 0 | 25 | 17 | 25 | 0 | 33 |
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 11 | 0 | 11 |

*ALP: Alkaline phosphatase positive intestinal metaolasia - A: Goblet cells with the gastric musosa*
*B: Intestinal type crypt without Paneth cells - C: Intestinal type crypt with Paneth cells.*

the X-ray groups were 84-95%, and in the non-irradiated groups were only 11-33%. The number of type B metaplasias and totals in the X-ray groups were significantly increased, and type C lesions were also more common in the X-ray + AOM and X-ray alone groups than in the controls (Table IV).

The first tumor appeared at 204 days, in an X-ray + AOM animal. Total tumors in the AOM groups were significantly more numerous than in the PhIP groups. Gastric tumors in the glandular stomach were observed in four out of the 25 (17%, three ATP, one adenocarcinoma) X-ray + PhIP animals, and four out of the 29 (10%, one ATP and three adenocarcinomas) X-rays + AOM animals. One signet ring cell carcinoma was found in this group. All other tumors in the glandular stomach were well-differentiated without goblet cells or mucin and were located in the middle or upper portion. Nuclei of

**Table IV** - Mean number of intestinal metaplasia

| Group | ALP | Pylorus | | | | Pylorus+Fundus | | | |
|---|---|---|---|---|---|---|---|---|---|
| Group | | A | B | C | Total | A | B | C | Total |
| X-ray+PhIP | 19.1±26.4 | 0.6±1.1 | 6.9±5.5* | 1.4±1.8 | 8.9±7.3* | 0.6±1.1 | 7.4±6.2* | 1.5±1.9 | 9.5±8.2** |
| X-ray+AOM | 25.0±42.2* | 0.4±0.8 | 8.4±7.7** | 2.0±2.2* | 10.9±9.3** | 0.4±0.8 | 9.1±7.5** | 2.4±2.2* | 11.9±8.7** |
| X-ray | 11.4±18.9 | 0 | 9.8±10.0** | 1.8±3.4 | 11.2±11.4** | 0 | 10.7±10.9** | 2.3±3.7* | 12.7±12.6** |
| PhIP | 0 | 0.1±0.3 | 0.8±2.6 | 0 | 0.8±2.6 | 0.1±03 | 1.1±2.8 | 0 | 1.2±2.8 |
| AOM | 0 | 0.5±1.4 | 0.2±0.6 | 0 | 0.7±0.6 | 0.5±1.4 | 0.4±0.8 | 0 | 0.9±1.5 |
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0.1±0.3 | 0 | 0.1±0.3 |

**: Significantly difference from Control value (P<0.05) - **: Significantly difference from Control value (P<0.01)*

intestinal metaplasia (Fig.4), and the signet ring cell carcinoma (Fig.5) were positive for CDX2 by immunohistochemistry. On the other hand, the cytoplasm of a well-differentiated adenocarcinoma was positive (Fig.6). No gastric tumors were observed in the other groups (Table V).

The incidence of small intestinal tumors was 28% and 3% in the X-ray+AOM and AOM groups, respectively. The more frequent colon tumors were the multiple and the papillary and polypoid types. The incidence of colon tumors was 8%, 12%, 79% and 72% in the X-ray + PhIP, PhIP, X-ray + AOM and AOM groups, respectively, and the number of tumors was 0.08 ± 0.28, 0.12 ± 0.3, 1.31 ± 0.97 and 1.17 ± 1.04, respectively (Table V). The incidences of signet ring cell carcinomas were 2 (7%) in the X-ray + AOM and 5 (19%) in the AOM groups. Aberrant crypt foci were observed in all of the chemical carcinogen-treated groups (data not shown).

Pancreas (17-38%) and skin tumors (6-28%) also developed after X-ray treatments. Three kidney tumors were found in the X-ray + PhIP group, three

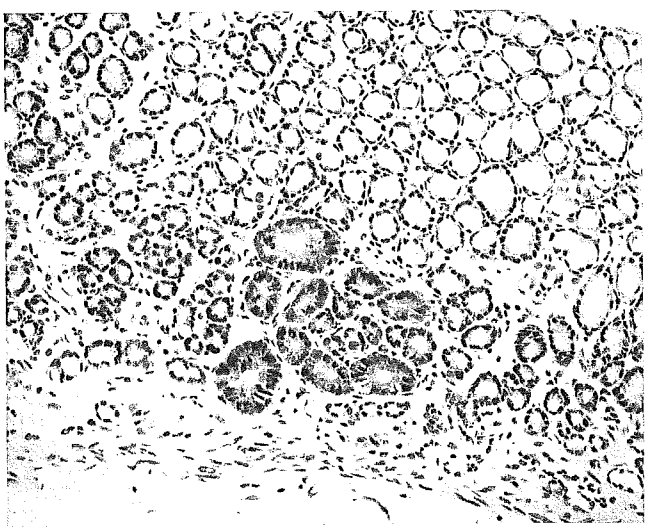

**Fig. 4** - CDX-2 positive nuclei were observed in glands of intestinal metaplasia, x100, CDX-2 antibody staining.

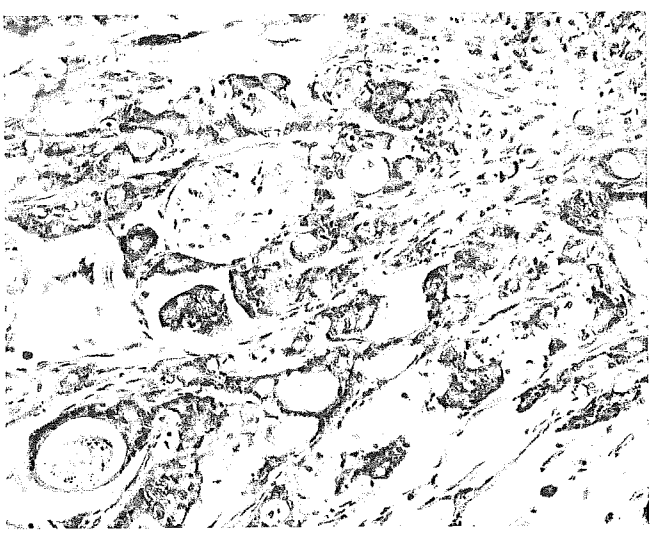

**Fig. 5** - CDX-2 positive nuclei were observed in signet ring cell carcinoma, x 200, CDX-2 antibody staining.

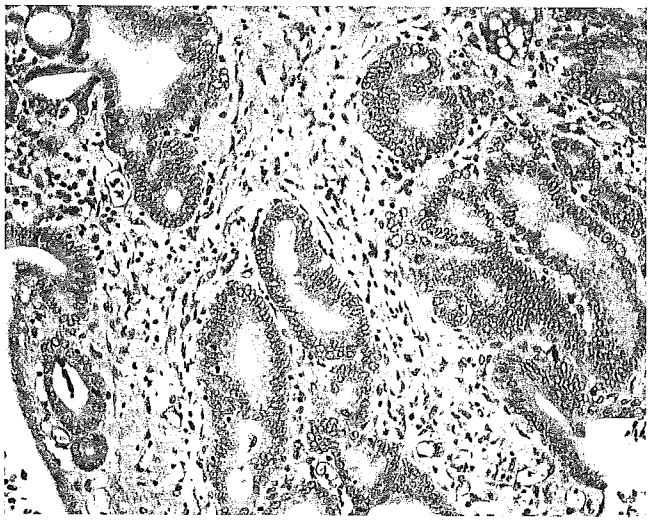

**Fig. 6 -** CDX-2 positive cytoplasm was observed in well differentiated adenocarcinoma x 200, CDX-2 antibody staining.

ear duct tumors, one bone and two liver tumors in the X-ray + AOM group, and one tail papilloma in the PhIP group (Table V).

## Discussion

In the present experiment, induction of intestinal metaplastic mucosa in the glandular stomach was associated with susceptibility to tumorigenesis induced by PhIP and AOM, in contrast to non-susceptible normal gastric mucosa. In earlier studies, regression analysis of gastric tumors per rat against the frequency of intestinal metaplasia, with or without Paneth cells, yielded a significant inverse relationship, suggesting that the development of intestinal metaplasia and gastric tumors might be independent (8,9). Previously, we reported that intestinal metaplasia is not susceptible to gastric tumor induction by MNNG and MNU (8,9) and that colonic mucosa transplanted into gastric mucosa lacks susceptibility to these carcinogens when given orally (24,25). Intestinal metaplasia or colorectal mucosa implants into the glandular stomach are sensitive to DMH carcinogenicity, whereas the normal gastric mucosa is not (10). Thus, it would appear that areas of intestinal metaplasia induced by X-irradiation might be susceptible to damage due to carcinogens targeting the large intestine. In the present experiment, CDX-2 appeared in intestinal metaplasia and in some of the gastric tumors. CDX-2 is not expressed in the normal stomach but is highly expressed in the normal intestine and intestinal metaplasia (23,27) and carcinoma of the stomach, indicating its involvement in these lesions. So, it is considered that some of the gastric tumors in this experiment might have been caused by intestinal metaplasia and/or circumstances

**Table V -** Incidence and number of colon tumors

| | No | Total (%) | Gatsric (%) | Small intestine (%) | Colon tumor Incidence (%) | Colon tumor Number per rat | Pancreas (%) | Skin (%) | Other (%) |
|---|---|---|---|---|---|---|---|---|---|
| X-ray+PhIP | 25 | 16(64) | 4(17) | 0 | 2(8) | 0.08±0.28 | 8(32) | 7(28) | 3(12) Kidney 3 |
| X-ray+AOM | 29 | 27(93) | 4(10) | 8(28) | 23(79) | 1.31±0.97 | 5(17) | 3(10) | 6(21) Ear duct 3 Liver 2 Bone 1 |
| X-ray | 32 | 15(47) | 0 | 0 | 0 | 0 | 12(38) | 2(6) | 2(6) Squamous cell carcinoma 1 Papilloma 1 |
| PhIP | 25 | 4(16) | 0 | 0 | 3(12) | 0.12±0.33 | 0 | 0 | 1 Tail papilloma1 |
| AOM | 28 | 23(79) | 0 | 1(3) | 21(72) | 1.17±1.04 | 0 | 0 | 2(9) Liver 1 Testis 1 |
| Control | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**:Significantly difference from X-ray group (P<0.05) - a: Significantly difference (P<0.05) - b: Significantly difference (P<0.01)*

of intestinal metaplasia.

We must consider the alternative possibility that the effects of irradiation and DMH and other colon carcinogens on glandular stomach epithelial cells are additive or synergistic. Tatemichi et al. reported that the cytochrome P450 monooxygenase 1A1 expressed in intestinal metaplasia, and carcinogen activation by 1A1 enzymes expressed in the gastric mucosa, may contribute to carcinogenesis of the stomach (28). It appears likely that intestinal mucosal stem cells are susceptible to colon carcinogenesis, independently of the administration route or their location. Thus, the intestinal mucosal phenotype appears to be the most important determinant of response to colon carcinogens, rather than the intestinal macro-environment itself.

In summary, the presence of intestinal metaplasia, with or without Paneth cells, may increase the sensitivity of the stomach to the induction of tumors by carcinogens like DMH, AOM or PhIP, but not by MNNG or MNU. The protocol used in the present experiment may provide a new approach for distinguishing between developmental events associated with intestinal metaplasia and gastric tumors.

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# Protective effects of a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia and *Agaricus blazei* murill against X-irradiation in B6C3F1 mice: Increased small intestinal crypt survival and prolongation of average time to animal death

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**Abstract.** Radioprotective effects of a water-soluble extracts from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia (designed as MAK) and *Agaricus blazei* (Agaricus) against the shortening of survival time or the injury of crypt by X-irradiation were investigated in male B6C3F1 mice. MAK and Agaricus at three different doses were mixed into basal diet into biscuits at 5, 2.5 and 1.25% and administered from 1 week before irradiation. MAK (5% group) significantly prolonged animal survival as compared with basal diet group (control group) after 7 Gy of X-ray irradiation at a dose rate of 2 Gy $min^{-1}$. At doses of 8, 10 and 12 Gy X-irradiation at a dose rate of 4 Gy $min^{-1}$ MAK (5% group) significantly increased crypt survival as compared to other groups. These results suggest that MAK can act as a radioprotective agent.

## Introduction

One major goal of radiobiology research is the development of drugs that can be used to provide protection against radiation injury, and numerous compounds have been developed and tested. The observed protective effects point to the possibility of improving the therapeutic index of cancer radiotherapy, or reducing the acute radiation effects in persons exposed in accidents. The strategy of reducing radiation injury to normal tissues might thus have significant benefit in terms of medical applications. Hsu *et al* (1-4) reported radioprotective effects of several kinds of Chinese traditional prescriptions and enhanced immunocompetence after irradiation was found. These results have encouraged us to search for other drugs that might exert radioprotective influence.

Various mushrooms have a long history of use in folk medicine, and become subjects of great interest, due to their multiple nutritional and pharmacological properties. Mushroom extracts are widely sold as nutritional supplements and touted as beneficial for health. However, only a few studies are available on the biological effects of mushroom consumption. *Ganoderma lucidum* (Fr.) Karst, belonging to the Basidiomycetes class of fungi, is colloquially known as 'Rei-shi' or 'Mannentake' in China and Japan, and it has been attributed with various medical virtues handed down in folklore. *Ganoderma lucidum* exhibits anti-hepatoxic and free radical scavenging activity (5), exerts influence on the cell cycle and cellular signal transduction (6), inhibits leukemic-cell growth (7), and induces differentiation of leukemic cells into mature monocytes/macrophages (8). In addition, it may inhibit platelet aggregation (9), impede complex interactions of viruses with cell plasma membranes (10), inhibit tumor growth (11) and decrease the incidence of mouse lung tumors (12). A water soluble extract from cultured medium *Ganoderma lucidum* (Rei-shi) mycelia (designed MAK) contains various kinds of high molecular constituents, i.e. polysaccharides with protein or water-soluble lignin, and low molecular constituents, i.e. triterpenes. Previously, we have reported that MAK prevented the development of azoxymethane induced aberrant crypt foci (ACF,13), development of N,N'-dimethylhydrazine-induced colon tumors in ICR mice (14) and colon tumors induced by azoxymethane in F344 rats (15).

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*Key words:* radiation protection, crypt survival, survival, *Ganoderma lucidum* (Rei-shi) mycelia, Agaricus blazei, B6C3F1 mouse

Table I. Change of testis 28 days after 6 Gy X-irradiation.

| Group | Size (μm) | Total no. | PCNA negative | Ratio (negative/ total) |
|---|---|---|---|---|
| X-ray+ 5% MAK[a] | 77.0±8.0 | 50.9±6.0 | 7.4±2.7[b] | 14.0±5.0[c] |
| X-ray+ 5% Agaricus | 70.0±8.0[c] | 45.0±6.1 | 12.6±5.4[b] | 28.0±11.0[c] |
| X-ray | 75.0±8.0[c] | 46.1±9.3 | 10.6±5.5 | 23.0±11.0 |
| Control | 175.0±14.0 | 29.0±3.8 | 0 | 0 |

[a]MAK, a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia. [b]$p<0.05$; [c]$p<0.01$.

The Basidiomycete mushroom *Agaricus blazei* Murrill, native to Brazil and popularly known in Japan as Himematsutake, has been largely produced and consumed as food and tea due to its medicinal effects, possibly including anti-carcinogenic activity (16,17). However, no experimental data exist regarding beneficial effects of this species of mushroom.

The present study was therefore conducted to assess the effects of MAK or *Agaricus blazei* extracts on crypt and animal survival after X-irradiation in mice.

## Materials and methods

*Animals.* Six-week-old male B6C3F1 (Crj:B6C3F1) mice and our standard protocol for assessing radiation effects were employed in the present experiment. Animals were housed in polycarbonate cages, five per cage, and kept under constant conditions of temperature (24±2°C) and humidity (50±10%) with a 12 h light/12 h dark cycle, according to the Guide for Care and Use of Laboratory Animals established by Hiroshima University, and fed a commercial diet MF (Oriental Yeast Co. Ltd., Tokyo, Japan) alone or with a 5, 2.5 and 1.25% supplement of MAK and Agaricus in biscuits. Normal tap water was also provided *ad libitum*.

*MAK and Agaricus.* A water-soluble extract from culture medium of *Ganoderma lucidum* mycelia (designed as MAK) was prepared by Noda Shokkin-Kogyo Co., Ltd. (Chiba, Japan). In brief, *Ganoderma lucidum* (Rei-shi or Mannnentake) mycelia were cultured in a solid medium composed mainly of sugar-cane bagasse for 3 months, then the whole medium containing mycelia was extracted with hot water. The extract was filtered and spray-dried as MAK. Agaricus was purchased as a commercial powder of *Agaricus blazei* Murill.

*Radiation.* Groups of mice were whole body irradiated with 6 or 7 Gy of X-rays (each 10 animals) at a dose rate of 2 Gy/min for the animal survival study and 8, 10 or 12 Gy of X-rays once for crypt survival (each 5 animals) at a dose rate of

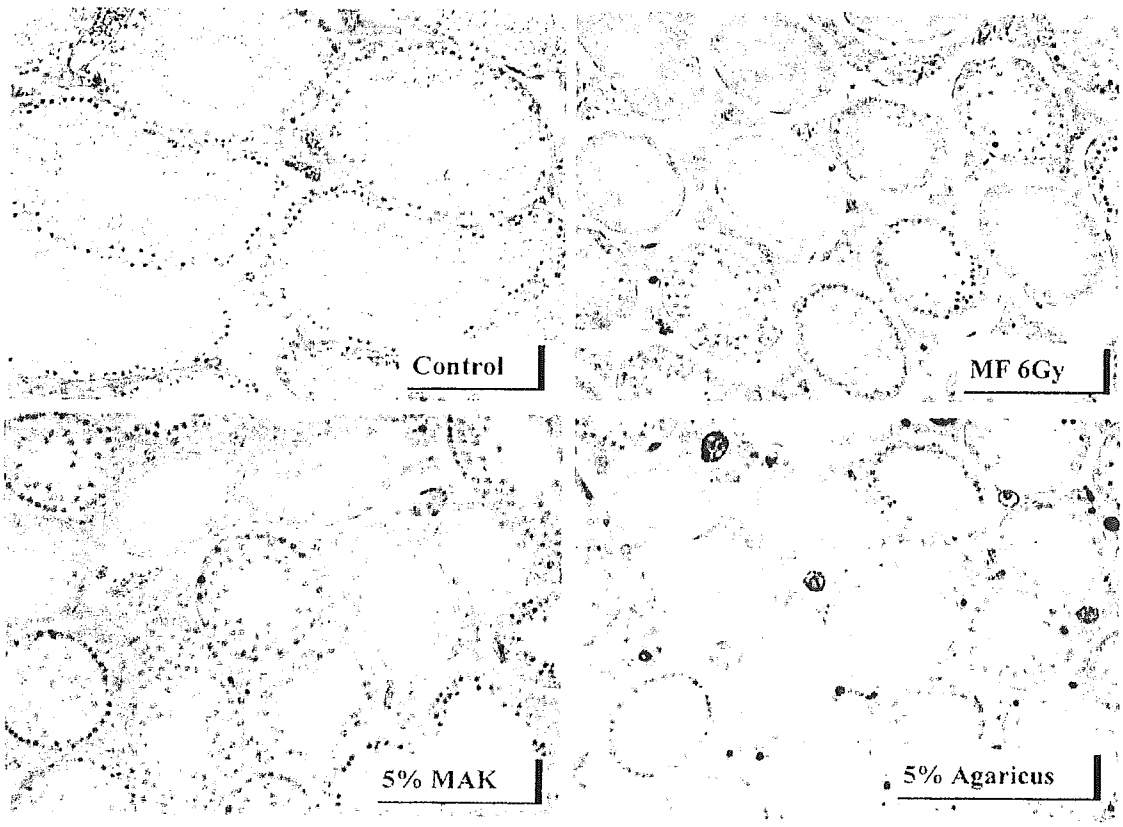


Figure 1. PCNA staining in seminiferous tubules.

Table II. Size of seminiferous tubules 4 weeks after 6 Gy whole body irradiation.

| Group | Size (μm) |
|---|---|
| MF | 75.0±7.8[a] |
| 5% MAK | 76.9±8.0 |
| 5% Agaricus | 70.3±8.5[a] |

MAK, a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia. [a]Significantly different from 5% MAK.

4 Gy/min as measured with a Radocon 555 dosimeter. The mice were not anaesthetized during the irradiation. Exposure factors were as follows: 200 kVp and a half-value layer 1.18 mm Cu. The X-ray air dose (in R) was then converted to the absorbed dose (in cGy) using a factor of 0.95 cGy/R.

One week before irradiation, the mice were given a diet supplemented with MAK and Agaricus and kept for 28 days on the same diet after X-irradiation with 6 and 7 Gy. The animals were observed every day at 8:00, 12:00 and 18:00, and deaths were recorded for the animal survival experiment. In the other groups, the animals were kept for 3.5 days after irradiation then sacrificed for determination of crypt survival.

*Autopsy.* Immediately after sacrifice, segments of the jejunum from the ileocecal junction (30 to 40 cm) were removed and fixed in Carnoy's solution. They were cut into several pieces, bundled together, embedded in paraffin, sectioned at a thickness of 3 μm and stained with hematoxylin-eosin. To quantitative regenerating crypts, number of crypts per circumference was determined in cross-section (18). In each mouse the number of surviving crypts in 10 gut cross-sections was scored.

Animals were sacrificed after cumulative irradiation for 28 days. Testes were fixed in FSA solution (37% formalin 5 ml, 5% sucrose solution 15 ml and acetic acid 0.8 ml) for 5 days, then embedded, sectioned and stained routinely. Sizes of seminiferous tubules were measured. For immunohistochemistry, paraffin-embedded sections were deparaffinized in xylene, and rehydrated through graded alcohols. A 0.05 M PBS buffer was used to prepare solutions and for washes between the various steps. Incubations were performed in a humidified chamber. Three μm-thick sections were treated for 30 min at room temperature with 2% BSA and incubated with primary antibodies against monoclonal mouse anti-proliferating cell nuclear antigen antibody (Dako-PCNA, PC 10, code No. M 879) for 1 h at room temperature. For each case, negative controls were performed on serial sections whereby incubation with the primary antibody was omitted. All slides were then exposed to the secondary antibody, biotinylated horse anti-universal-monkey IgG (Vectastain Universal Quick Kit, Vector Laboratories, Ca, Catalog No. PK-8800) and peroxidase conjugated streptoavidin complexes. Peroxidase activity was visualized by treatment with $H_2O_2$ and diaminobenzidine for 5 min. At the last step, the sections were counterstained with hematoxylin for 1 min. PCNA-positive cells in seminiferous tubules were counted.

*Statistics.* Statistical significance was determined with Dunnett's method and the Cox proportional hazard model for multiple comparisons using logarithmic transformation and the Student's t-test.

## Results

Survival was not significantly affected with 6 Gy irradiation. Testes of surviving animals after 28 days of irradiation demonstrated significantly smaller seminiferous tubules in the 5% Agaricus group than with X-rays alone (Table I). The

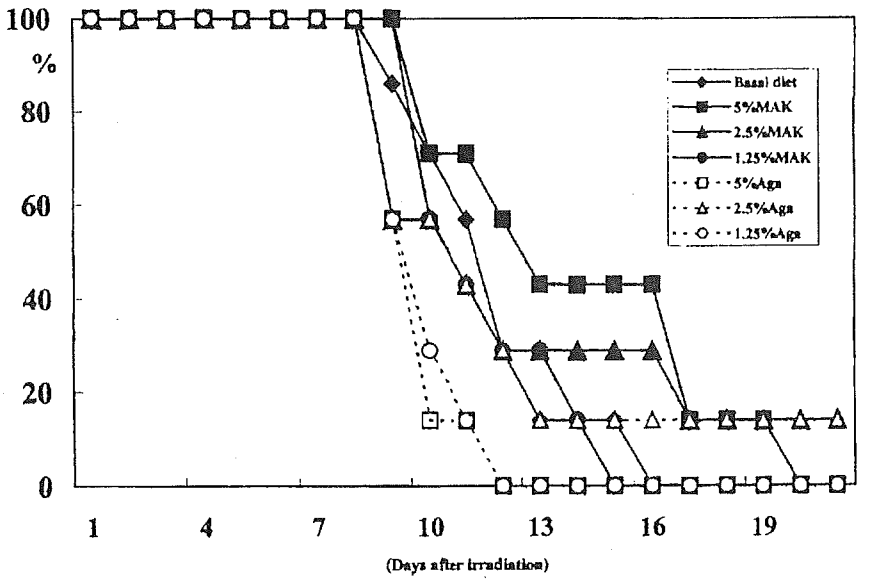


Figure 2. Survival after 7 Gy irradiation. MAK, a water-soluble extract at 5% from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia. MAK vs. Basal diet $p<0.02$, 5% MAK vs. 1.25% MAK $p<0.02$, 5% MAK vs. 5% or 1.25% AGA $p<0.007$.

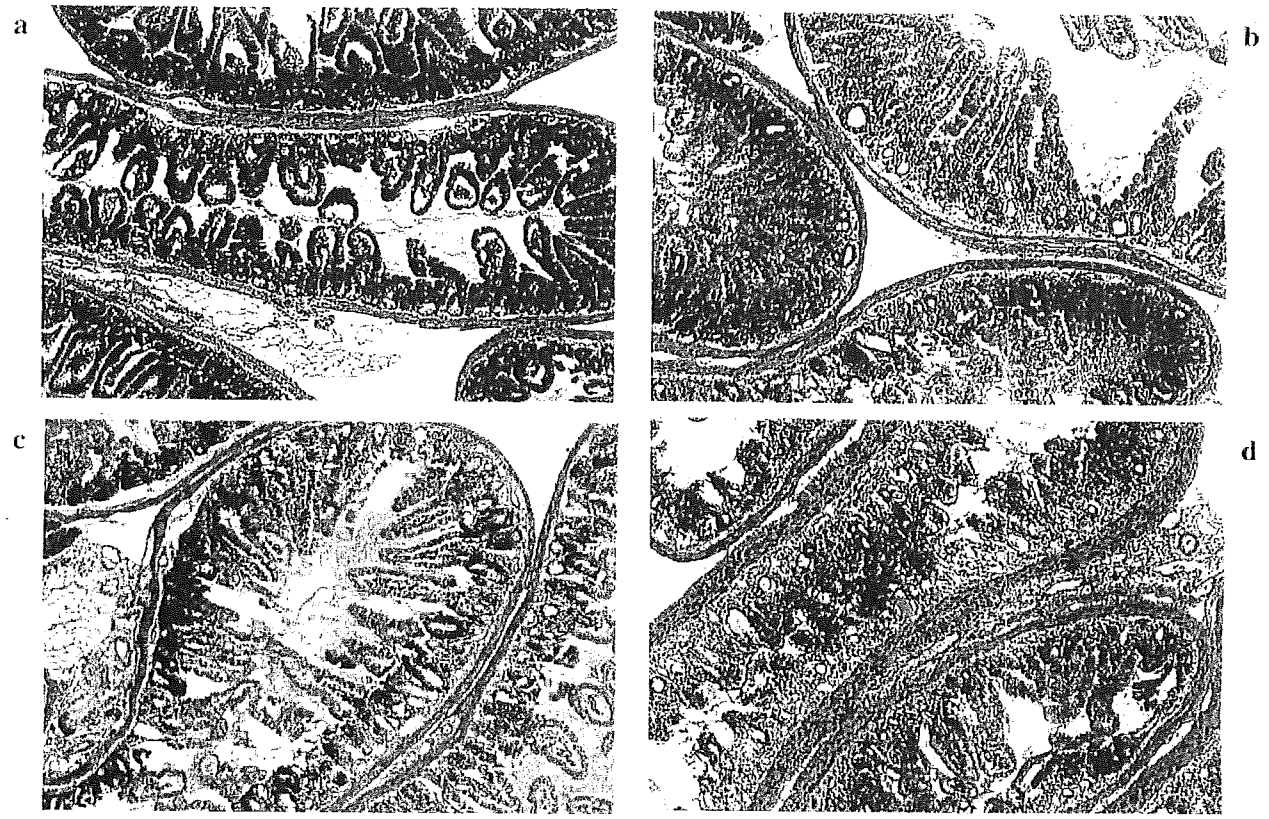


Figure 3. (a) Normal small intestine; (b) 10 Gy-irradiated small intestine in MF diet group. A few regenerated crypts were observed; (c) 10 Gy-irradiated small intestine in 5% MAK group. Many regenerated crypts were observed; (d) 10 Gy-irradiated small intestine in 5% Agaricus group. A few regenerated crypts were observed.

Table III. Crypt survival.

| | 0 Gy | 8 Gy | 10 Gy | 12 Gy |
|---|---|---|---|---|
| MF | 116.53±13.39 | 84.54±11.74 | 43.74±8.42 | 24.76±5.62 |
| 5% MAK | | 117.00±12.47 | 68.06±9.63 | 43.77±7.64 |
| 2.5% MAK | | 81.38±10.41 | 52.52±8.90[a] | 28.08±5.49[a] |
| 1.25% MAK | | 87.88±11.21 | 51.72±7.59[a] | 29.78±4.44[a] |
| 5% Agaricus | | 81.06±10.06 | 42.50±6.60 | 27.52±4.68[a] |
| 2.5% Agaricus | | 83.52±10.18 | 49.98±7.30[a] | 26.38±3.85 |
| 1.25% Agaricus | | 82.60±10.47 | 51.78±8.29[a] | 27.02±4.98 |

MAK, a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia. 5% MAK was significantly different from other groups; ($p<0.01$). [a]Significantly different from MF group ($p<0.05$). [b]Significantly different from MF group ($p<0.01$).

number of PCNA-negative seminiferous tubules was zero in control animals (Fig. 1). Ration of PCNA negative vs. total seminiferous tubules in 5% MAK values was significantly smaller than that in 5% Agaricus values (Table II). Animals in Agaricus groups started to die 9 days after irradiation and survival is shown in Fig. 2. Delay in mortality was evident in 5% MAK group, with significantly increased survival in the MF ($p=0.02$), 1.25% MAK ($p=0.02$) and 1.25% Agaricus ($p=0.007$) by the Cox model.

The number of crypts in one circumference in the non-irradiated group was 116.5±13.4 (Fig. 3a). A dose-dependent decrease was evident with 8-12 Gy (*Table III* and Fig. 3b) and surviving crypts in 5% MAK (Fig. 3c) were significantly increased, compared to other groups in every dose. Crypt survival was evident with a significant difference in 2.5 and 1.25% MAK and Agaricus (Fig. 3d) ($p<0.01$) as compared with MF group in 10 Gy irradiation and in 2.5%, 1.25% MAK ($p<0.01$) and 5% Agaricus ($p<0.05$).